# Designed Ankyrin Repeat Proteins for Targeted Cancer Therapy

Dissertation

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*For*

*My husband Rolf*

*And my son Manuel*

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# Zusammenfassung

Neue Generationen von bindenden Molekülen, die nicht auf dem Immunglobulin-Gerüst basieren, sind zu einer attraktiven Alternative zu Antikörpern geworden. Während die Spezifität von Antikörpern erhalten bleibt, bieten diese neuen Generationen von Bindungsproteinen mehrere Vorteile gegenüber Antikörper in Bezug auf molekulare Robustheit als Voraussetzung für z.B. Affinitätsmaturierung, sowie Stabilität und Wirtschaftlichkeit der Produktion. Ein Beispiel für eine solche Alternative sind Designed Ankyrin Repeat Proteine (DARPin). Der Schwerpunkt dieser Arbeit war die Generierung von spezifischen DARPins, die das tumorassoziierte Antigen EpCAM (epithelial cell adhesion molecule) erkennen, und deren Verwendung als Trägersubstanz für ein Biotoxin zur tumorgerichteten Therapie. Die Wahl von EpCAM als Zielstruktur beruht auf dessen vorteilhaften Eigenschaften als Bindungsstellen auf Tumorzellen wie zum Beispiel die Überexpression in zahlreichen soliden Tumoren, der geringen Expression und anatomisch bedingten schlechteren Zugänglichkeit in normalen epithelialen Geweben, der stabilen Expression ohne "shedding" und der Fähigkeit, nach Ligandenbindung in die Zelle zu internalisieren. All diese Eigenschaften machen EpCAM zu einer idealen Zielstruktur für die tumorgerichtete Behandlung mit hochzytotoxischen Krebsmedikamenten einschliesslich Biotoxine, die ohne diese Tumorselektivität nicht anwendbar sind. Das günstige therapeutische Fenster EpCAM-spezifischer Krebstherapien wurde bereits in früheren Studien bestätigt.

Der erste Schritt zum spezifischen EpCAM Targeting war die Selektion und Charakterisierung von DARPins, die gegen die extrazelluläre Domäne von EpCAM gerichtet sind. Phage-Display- und Ribosome-Display Methoden wurden angewandt, um eine erste Generation von Bindern gegen EpCAM zu erhalten, die anschliessend durch Affinitätsreifung noch weiter verbessert wurden. Die zweite Generation von Bindern wurde charakterisiert, und die sechs besten EpCAM-spezifischen DARPins wurden ausgewählt. Sie waren sehr gut exprimierbar, monomerisch und erkannten nicht nur das lösliche EpCAM, sondern konnten auch im zellulären Kontext an EpCAM binden. Basierend auf der Affinität, wählten wir einen dieser Binder, EC4, zur weiteren Analyse als Targeting-Einheit für die EpCAM-gerichtete Krebsbehandlung mit einem hochgiftigen Biotoxin.

Hierfür wurde Ec4 C-terminal mit einer verkürzten Form des Pseudomonas Exotoxin A (ETA) ohne dessen bindende Domäne fusioniert. Ec4-ETA" war löslich und in grossen Mengen im Zytoplasma von E. coli exprimierbar. Der Vorteil der Verwendung eines DARPins für die Generierung des Fusionsproteins wird dadurch verdeutlicht, dass dieses Fusionstoxin zu einer 50 mal höheren Ausbeute und wesentlich vereinfachter Aufreinigung führte, verglichen mit einem EpCAM-spezifischen scFv, der mit ETA" fusioniert ist und im Periplasma exprimiert wurde. Nach biochemischer Charakterisierung des DARPin-ETA'' Fusionsproteins wurde die Zytotoxizität gegen Tumorzellen *in vitro* und *in vivo* analysiert. EC4-ETA" erwies sich als hochzytotoxisch gegen verschiedenen humane Krebszellen, dabei war dieser Effekt abhängig von der Expression von EpCAM. So wurde nur eine sehr geringe Hintergrundaktivität gegen EpCAM-negative Zellen gemessen und die Zytotoxizität von Ec4ETA" wurde auch durch Zugabe von löslichem EC4 DARPin als Kompetitor gehemmt, nicht aber durch Zugabe eines unspezifischen DARPins. In vivo Fluoreszenz-Bildgebung in Nacktmäusen mit subkutan wachsenden HT29 Kolonkarzinomen zeigte, dass sich EC4-ETA" in Tumoren ansammelt mit Maximalwerten 48 bis 72 Std. nach intravenöser Injektion, während eine unspezifische DARPin ETA"-Fusion keine Tumorlokalisation zeigte. Dieses günstige Tumorlokalisierungsprofil korrelierte direkt mit der Antitumoraktivität des Fusionstoxins, so kam es nach Injektion von Ec4-ETA" in gut verträglicher Dosierung zu einer deutlichen Hemmung des Wachstums der Kolonkarzinome mit vereinzelt sogar kompletten Regressionen.

Zusammenfassend haben wir erstmals hochaffine und stabile EpCAM-spezifische DARPins hergestellt und charakterisiert. Am Beispiel einer hochzytotoxischen DARPin-Biotoxin Fusion konnten wir zudem deren Eignung für die tumorgerichtete Krebstherapie *in vitro* und *in vivo* demonstrieren.

## Summary

New generations of binding molecules not based on the immunoglobulin scaffold have become an attractive alternative to antibodies. While keeping the same affinity and specificity of antibodies, they can provide several advantages over them concerning robustness, stability and economy of production. An example of such an alternative scaffold is the Designed Ankyrin Repeat Protein (DARPin). The focus of this thesis was on the generation of specific DARPins recognizing the clinically validated tumor associated antigen epithelial cell adhesion molecule (EpCAM) and their use as a vehicle for the delivery of a highly potent biotoxin. The choice of EpCAM as a target was based on its favorable characteristics as a tumor associated antigen such as overexpression in numerous tumor types, low expression and poor accessibility on normal tissues, no considerable shedding or downregulation and its ability to internalize upon ligand binding. All these characteristics make EpCAM an ideal target for the tumor-targeted delivery of cytotoxic payloads. Moreover, the reasonable therapeutic window offered by EpCAM targeting has been demonstrated by several approaches.

The first step to achieve highly specific targeting of tumor cells was the selection and characterization of DARPins against the extracellular domain of EpCAM. Phage display and ribosome display were applied to obtain a first generation of EpCAM binders which were then further improved by affinity maturation. The second generation of improved binders was characterized and the six best EpCAM-specific DARPins were chosen. They could prove to be well expressing monomers and recognized EpCAM not only in soluble form, but also bound to epitopes accessible in the cellular context.

Based on the affinity, we chose one of these binders, Ec4, for further analysis as a vehicle for targeted delivery of a highly potent biotoxin to human tumor cells. To this end, Ec4 was C-terminally fused to a truncated form of Pseudomonas exotoxin A (ETA") lacking the cell binding domain. Ec4-ETA" was expressed in soluble form and at high yields in the cytoplasm of *E. coli*. The advantage of using a DARPin for the generation of the fusion protein was thus convincingly demonstrated by a 50-fold higher production yield, and more simplified purification steps compared to its counterpart, an EpCAM-specific

scFv-ETA" immunotoxin, which in soluble form only expressed in the periplasm. Upon biochemical characterization, the DARPin-ETA'' fusion protein was analyzed for cytotoxicity and antitumor activity *in vitro* and *in vivo.* Ec4-ETA" was highly cytotoxic to a EpCAM-positive tumor cell lines of various histotypes. This effect was EpCAM-dependent, as cytotoxicity was reduced to background levels on EpCAM-negative cells and further analysis revealed that it was also abolished upon addition of soluble Ec4 as competitor, but not an irrelevant unspecific DARPin. *In vivo* fluorescence imaging in nude mice bearing human HT29 colon carcinoma xenografts demonstrated that Ec4-ETA" accumulated in tumors with peak levels achieved 48 h to 72 h after injection, whereas an irrelevant unspecific DARPin-ETA fusion did not show tumor localization. The favorable tumor targeting of properties of Ec4-ETA" also translated into potent antitumor activity resulting in a strong inhibition of tumor growth at well-tolerated doses with some mice showing even complete regressions.

In conclusion, we generated and characterized for the first time EpCAM-specific DARPins of high affinity and stability, and in the form of a second generation fusion toxin as example demonstrated their potential for use in tumor targeting and therapy.

**Chapter 1**

# Introduction
# & Aim of the thesis

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# Cancer

Cancer remains the second most common cause of death in our society. Approximately 10 million people worldwide are diagnosed with cancer each year. There are more than 100 distinct types of cancer and within them, subtypes of tumors can be found. Lung and colorectal cancers are the most common cancers in both men and women, followed by breast cancer in women, and prostate cancer in men (Figure 1). In addition, metastatic cancers constitute the main threat of cancer to life and its management remains one of the most challenging aspects for oncologists.

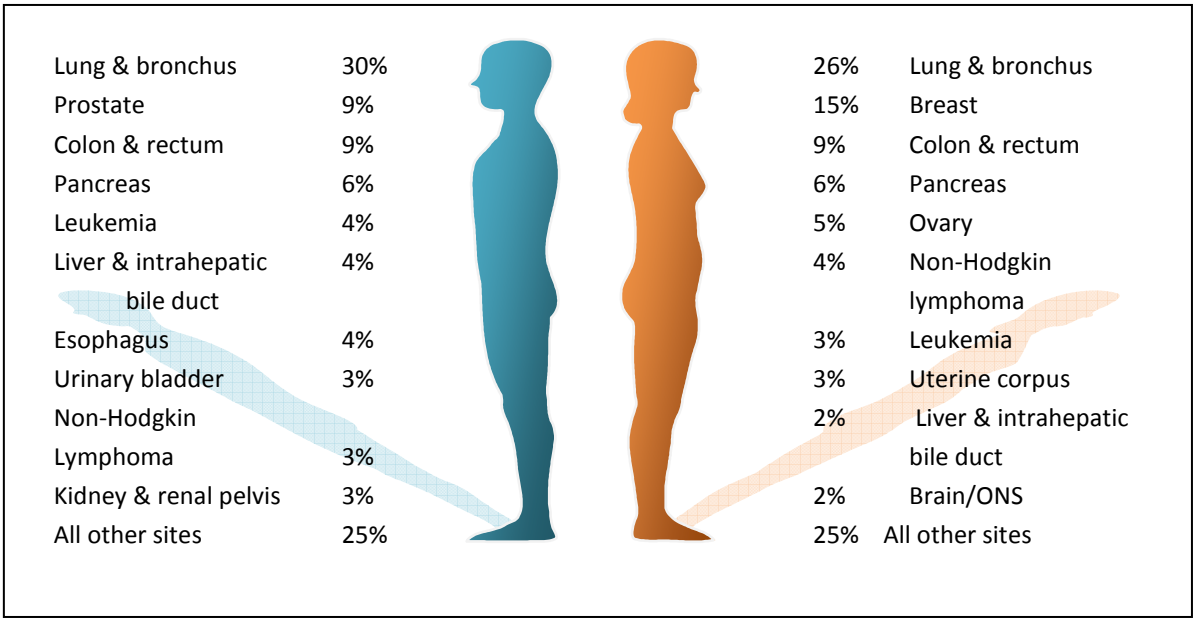



Figure 1. **Ten Leading Cancer Types, 2009** (1) **.** Lung cancer is the most common fatal cancer in men followed by prostate, and colon & rectum. In women, lung, breast, and colon & rectum are the leading sites of cancer death. ONS: other nervous system.

Cancer is a genetic disease arising from mutations in regulatory genes which result in a loss of growth control and consequently confers a survival advantage to the tumor cells. Cancer develops as a multistep process in which a succession of genetic changes leads to progressive conversion of a normal human cell into a cancer cell (2). As described by Hanahan and Weinberg (3), there are six essential alterations in cell physiology acquired during tumor development. These hallmarks are: the ability to proliferate independently of growth signals, the avoidance of programmed cell death (apoptosis), the insensitivity to growth suppressive factors, the ability to stimulate and sustain angiogenesis, the ability to invade tissue and to metastasize, and their limitless replicative potential. Recently, the inflammatory microenviroment around the cancer cell has been included as the seventh hallmark of cancer (4).

# Cancer treatments

## Conventional therapies

Conventional therapies to treat cancer such as surgery, radiation and chemotherapy have served well during many years in the battle against cancer. Chemotherapeutic drugs either alone or in combination with radiotherapy have improved the outcome of several solid or hematologic tumors. However, in spite of continuous advances, these therapies are limited and not without significant side effects.

Surgery is only suitable when treating cancer in localized areas or when tumors are accessible, but not when tumors have already spread through the body. Radiation does not discriminate between tumor cells and healthy tissues. It destroys all cells in the area being treated and relies on externals methods to find the desired location and to minimize harm to nearby healthy tissues.

Chemotherapy interferes with the ability of rapidly dividing cells to grow, a characteristic of cancer cells. However, healthy cells are also affected, especially those rapidly dividing cells like the skin, the lining of the stomach, the intestines and bone marrow. Therefore, the major limitation of these therapies is the lack of tumor specificity and toxicity to normal tissues. Suboptimal doses have to be given, leading to failure of the therapy or to drug resistance. To overcome the limitations of conventional therapies there is a need to develop new cancer treatments.

## Targeted therapies

The greater understanding of mechanisms of how cancer cells operate, how they become malignant and of their antigenic profile contributes greatly to the design of therapies that target tumors more specifically. The identification of factors that influence the carcinogenic process led to the discovery of a number of critical steps that can be targeted for therapeutic intervention. As a result, normal cells are spared and thus very little associated toxicity is expected. Several types of targeted therapies are under development and some of them haven been approved for cancer treatments (5). Some of these approaches include: small molecule drugs with greater specificity (6), cancer vaccines (7), gene therapy (8), drug carriers such us liposomes or nanoparticles (9), and ligand or antibody-based therapies (10) (Figure 2).

Among the targeted therapies, antibody-based therapies have become a major strategy in clinical oncology. In general, this targeting system consists of two parts:

- The tumor antigen which is the targeted molecule.
- The targeting moiety: mostly an antibody or antibody fragment, but growth factors, cytokines and ligands are also used. This moiety may act by itself or may serve as a vehicle for the deliver of a cytotoxic agent.

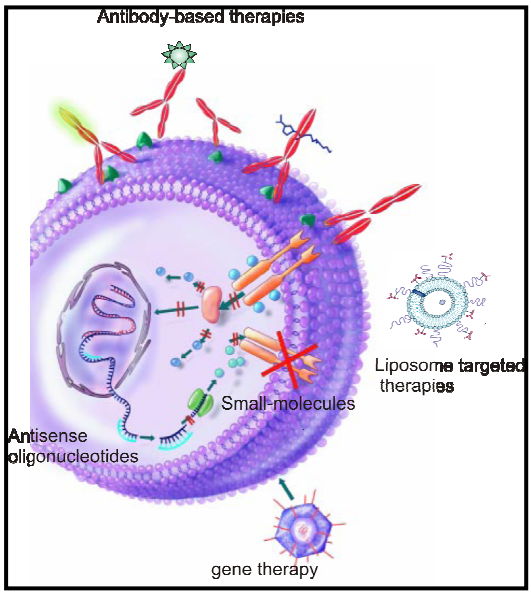



Figure 2. **Different types of targeted cancer therapies**. Antibody based therapies: unconjugated antibodies can produce their effect either by blocking receptors or marking the cells to be recognized by the immune system. Antibodies can also serve as vehicles to deliver antitumor agents, such as radioisotopes, toxins or cytotoxic compounds. Another approach is to inhibit signaling pathways important for tumor growth by small-molecules drugs. Gene therapy holds promise for blocking expression of oncogen or repairing of tumor repressor genes. Antisense oligonucleotides can block protein synthesis by binding to specific mRNA sequences. Selective targeting of ligand-targeted liposomes and nanoparticles containing anticancer drugs is another strategy to improve the therapeutic effectiveness of these drugs. Figure adapted from Segota et al. (5).

## The importance of the targeted antigens

The therapeutic efficacy of the drug used in a targeted therapy depends in great part on the properties of the targeted antigen. Due to tumor heterogeneity and similarity to normal tissues, identification of truly specific tumor antigens is a very difficult challenge. Most of the characterized antigens

are considered to be associated to tumors but to some extent are also present on normal cells. Tumor-associated antigens (TAAs) are mostly derived from mutated or differentially expressed proteins between tumor and normal cells. Characteristics that make a TAA an ideal candidate for targeted therapy include:

- High level and frequency of expression on tumor cells and low expression or inaccessibility on normal cells.
- No loss or reduction of antigen expression during tumor development and progression or by chemotherapeutic treatments
- Absence of substantial antigen shedding
- Accessibility to the targeted drug

Many TAAs have been identified and characterized (11). They are used in the detection, diagnosis and treatment of a variety of cancers. Some examples are summarized in Table 1. Among them, one promising TAAs which fulfills the criteria to be used for targeted therapies is the epithelial cell adhesion molecule (EpCAM) and will be described more in detail.

## EpCAM

EpCAM is a 40 kDa transmembrane glycoprotein consisting of an extracellular domain of 246 amino acids, a 23 amino acid transmembrane domain and a short intracellular domain of only 26 amino acids. The extracellular part of EpCAM comprises an epidermal growth factor (EGF)-like domain and a human thyroglobulin (TY)-like domain followed by a cysteine-poor region (23-25).

Table 1. Some examples of TAAs used for the detection, diagnosis and treatment of different cancers

| TAAs | Overexpression in |
|---|---|
| CEA | Several solid tumors: colorectal, gastric, pancreatic, NSCLC, esophageal and breast cancer (12) |
| CA125 | ovarian cancer (13) |
| PSCA | prostate cancer (14) |
| HER2/neu | Several solid tumors: breast, ovarian, gastric and colorectal carcinomas (15) |
| MUC-1 | colon and breast cancer (16) |
| EGFR | Several solid tumors: non-small-cell-lung cancer (NSCLC), prostate, breast, stomach, colon, ovary and head and neck (17-18) |
| EpCAM | Several solid tumors: colon, breast, NSCLC, head and neck, bladder, and prostate cancer (19-21) |
| CD25 (IL2-receptor), CD22 | Hematological malignancies (22) |

Abbreviations used: CEA: carcinoembryonic antigen, CA-125: cancer antigen 125, PSCA: prostate stem cell antigen, HER2/neu: human epidermal growth factor receptor 2, MUC-1: mucin 1, EGFR: epidermal growth factor receptor, EpCAM: epithelial cell adhesion molecule, CD: cluster of differentiation, IL: interleukin

EpCAM was discovered more than 30 years ago (26); its function was proposed as a calcium-independent cell adhesion molecule (27). It was shown that it can interfere with the function of other cell adhesion molecules. Regulation of E-cadherin junctions may be one of the pathways by which EpCAM affect cell differentiation and promote cell invasion (28). A hypothetical model proposed for the structure of EpCAM mediated adhesions in the cell membrane is the formation of tetramers with lateral and reciprocal interactions (29) (Figure 3). But restricting EpCAM function to be only a cell adhesion molecule could not

explain its increased expression in several tumors and its negative correlation with survival prognosis (30-31). Recently, EpCAM was described as a "surface-to-nucleus missile" (32) to explain its involvement in nuclear signaling. EpCAM is cleaved via regulated intramembrane proteolysis (RIP) by two enzymes, the tumor necrosis factor-alpha-converting enzyme and a gamma-secretase complex. The released cytoplasmatic domain induces the transcription of c-myc, cyclins and genes related to proliferation. Activation of EpCAM was found by soluble EpCAM or in zones of cell-cell contact, indicating that its oligomerization might trigger activation (33-35). Thus, it seems clear now that EpCAM has a dual role, both as an adhesion molecule as well as an oncogenic signal transducer. In addition, recent studies have identified a number of proteins that directly or indirectly interact with EpCAM, such as CD44v4-v7, and claudin 7 (36-39). Some of these interactions appear also to be involved in processes that promote carcinogenesis.

The presence of high levels of EpCAM has been well defined in a variety of solid tumors such as colon, breast, prostate, head-and-neck, lung and others (19-21, 40-42) and for this reason is considered one of the most frequently expressed TAA. Although it is expressed in some normal epithelial tissues, it is mostly located on the basolateral cell surface (23, 43). This location makes EpCAM in normal tissues less accessible than in tumor tissues where it is homogenously distributed (44). Moreover, EpCAM overexpression usually correlates with a decreased survival representing a negative prognostic marker for patients with breast, ovarian, gall bladder and head and neck tumors (30, 42, 45-46)

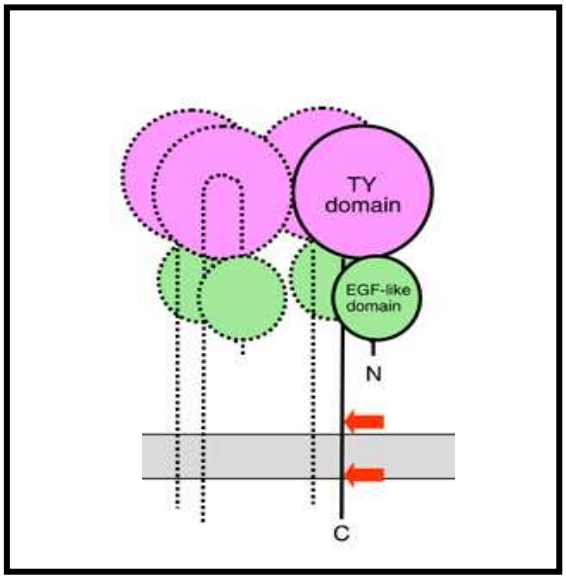



Figure 3. **Revised model of EpCAM on the cell membrane** (47). EpCAM is shown as a tetramer and each polypeptide chain is depicted with a bend within the TY domain and the EGF-like domain is oriented to the membrane. The cleavage site of EpCAM proteases are shown with arrows in red.

New data reveal that in several types of cancer, only a subset of cancer cells is capable of initiating tumor growth. This subpopulation of cells is defined as cancer stem cells (CSCs). They have the ability to self-renew and to differentiate, thereby generating the primary tumor and to disseminate to produce metastatic disease (48). Recently, EpCAM has been also identified on CSCs from a variety of cancer types such as breast, colon and pancreas (49-52). The concept of CSCs has a profound implication for cancer therapies (53): if the tumor growth is driven and maintained by CSCs, a major goal of cancer therapy will be to identify and kill these cells. Consequently, markers allowing the identification and of CSCs will become of great importance as CSCs usually respond poorly to standard chemotherapy.

Currently, at least seven candidate therapeutics focus on EpCAM as a target and are under clinical development (47). Many other approaches are under pre-clinical evaluation. Some of these approaches involve monoclonal and bi/tri specific antibodies (54-55), vaccination approaches (56), toxin fusion proteins (57-58), and drug-filled targeted liposomes (59).

In summary, the favorable properties of EpCAM as a tumor-associated antigen, its role in promoting cell proliferation, its presence on CSCs, and the increasing numbers of EpCAM-directed therapies which are under development, strongly support its use as a therapeutic target antigen for the eradication of both differentiated and pluripotent cancer cells.

## Antibodies as the tumor recognition moiety

For tumor targeted therapies monoclonal antibodies (MAbs) can be used in two different ways: as unconjugated molecules (naked antibodies), or coupled to effector molecules such as radionuclides, enzymes, drugs, cytokines, toxins and drug-filled liposomes or nanoparticles (60-61). Consequently, their mechanisms of action depend on how the antibody is used, the antibody itself and the targeted antigens.

### Unconjugated or naked monoclonal antibodies

Antibodies alone are usually not responsible for killing tumor cells. They bind to TAAs thereby either initiating signaling mechanisms that induce apoptosis in tumor cells, or marking tumor cells. Subsequently, the immune system recognizes tumors cells to which antibodies are bound and Fc domain-based functions are activated such as antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent complement mediated cytotoxicity (CDC). Antibodies can also bind to a specific epitope, thereby blocking the binding of the natural ligand preventing growth-promoting signals. Another example of how antibodies can be used therapeutically is the binding to a soluble growth promoting factor such as the vascular endothelial growth factor preventing the formation of new vessels in the tumors (62). Unconjugated antibodies approved

by the U.S. Food and Drug Administration (FDA), their targets, indication and their probable mechanisms of action are summarized in Table 2.

Although unconjugated MAbs show some therapeutic potency, they are seldom curative by themselves. Mostly they are used in combination with chemotherapeutic agents to increase the response rate and the efficacy of the therapy (63). One strategy to increase the therapeutic potency of antibodies is to use them as a transport vehicle for more potent anticancer drugs (10, 64).

Table 2. Unconjugated monoclonal antibodies used for cancer treatment (FDA approved)

| **Antibody** | **Brand name** | **Target** | **Probable mechanisms of action** | **Indicated for** |
|---|---|---|---|---|
| Rituximab | Rituxan® | CD20 | Triggers immune response and induction of apoptosis by crosslinking | B-cell non-Hodgkin's lymphoma |
| Trastuzumab | Herceptin® | HER2 | Induction of the immune system and prevention of growth-promoting signaling | Breast cancer |
| Cetuximab | Erbitux® | EGFR | Inhibition of signal transduction | Squamous cell carcinomas of the head and neck and colorectal cancer |
| Panitumumab | Vectibix® | EGFR | Inhibition of signal transduction | Metastatic colon cancer |
| Alemtuzumab | Campath® | CD52 | Triggers immune response | B-cell chronic lymphocytic leukemia |
| Bevacizumab | Avastin® | VEGF | Block angiogenesis | Colorectal cancer |

Abbreviations used: EGRF: epidermal growth factor receptor, HER2: human epidermal growth factor receptor 2, VEGF: vascular endothelial growth factor.

## Monoclonal antibodies as delivery vehicles for cytotoxic drugs

The rationale for using antibodies as delivery vehicles for toxic payloads is to combine the power of potent cytotoxic agents with the specificity of antibodies. These cytotoxic drugs, which are too potent to be used alone, can be selectively delivered to tumors using antibodies as targeting moieties. The main goal is to increase the amount of drug at the tumor site and decrease them at the healthy tissue. In addition, antibodies which are tumor specific but too little toxic to tumors, can acquire a higher toxic potential when fused to cytotoxic compounds. Accordingly, cytotoxic drugs, cytokines, radionuclides, enzymes and toxins have been conjugated to antibodies and evaluated in preclinical and clinical settings. At present there is one FDA-approved immunoconjugate containing a cytotoxic drug: gemtuzumab (Mylotarg®), consisting of a humanized anti CD33 mAb conjugated to the potent antibiotic calicheamicin (65). Two radiolabeled mAbs have been approved for the treatment of B-cell lymphomas: a CD20-specific antibody radiolabeled with $^{90}$Y (ibritumomab tiuxetan, Zevalin®) (66) and a CD20-specific antibody radiolabeled with $^{131}$I (tositumomab, Bexxar®)(67-68). Additionally, in 1999 the FDA approved the used of the first toxin conjugate: denileukin difitox, Ontak® for the treatment of patients with persistent or relapsed CD25-positive cutaneous T-cell lymphoma (69). Ontak is composed of the full length IL-2 ligand fused to a truncated form of Diphteria toxin. Targeting tumors using toxins fused to antibodies is a promising strategy to enhance antitumor activity of antibodies.

## Antibodies conjugated to toxins: immunotoxins

Toxins are really potent cytotoxic agents requiring only few molecules to kill a cell. Their potency is based on their catalytic activity with a high turnover rate, robustness to survive proteolytic degradation and an ability to interact with essential components of most cell types (70). Due to these properties, toxins produced by bacteria (e.g., Diphtheria toxin, *Pseudomonas* exotoxin A), or by

plants (e.g., gelonin, ricin, abrin) are used as anticancer agents (71). However, used alone they are toxic to many cells in the body. To widen their therapeutic window, they need to be targeted to cancer cells. To this purpose, TAA-specific antibodies can be conjugated to a toxins and redirect the cell-killing activity specifically to cancer cells only. These chimeric proteins, consisting of a targeting moiety, most frequently antibodies but also growth factors or interleukins, and a toxin moiety, are termed immunotoxins (ITs).

The first generation of ITs was developed by chemically conjugating whole toxins to antibodies (72) . Although they demonstrated the feasibility of the concept, they were ineffective due to a lack of specificity: toxins could still bind to healthy cells and thus kill them. Moreover, they showed poor stability and heterogeneous composition. In the second generation of ITs, by applying knowledge of toxin structures the cell-binding domain of the toxin was removed before conjugation with antibodies. This reduced the unspecific toxicity to normal cells but as they were still made by chemical coupling, heterogeneity remained a problem. The third generation has overcome this problem by making use of the advances in recombinant DNA techniques. Mostly antibody fragments, scFv or dsFv, are genetically fused to truncated form of the toxins. Thus, it is possible to generate homogenous products and also decrease the size of the ITs by using small antibody fragments, thereby improving tumor penetration.

A large number of clinical trials have been conducted with ITs over the last years. These ITs recognize a wide variety of TAAs present on hematological malignancies and solid tumors (71). However, immunogenicity and unspecific toxicity represent the two principal challenges that limit the effectiveness of ITs. Antibodies or antibody fragments are usually humanized; therefore, the immunogenicity is in most cases related to the toxin part of the IT. As toxins are foreign proteins, the human immune system may develop neutralizing antibodies against them. The binding of antibodies to ITs results in loss of efficacy and precludes repeated treatment, limiting ITs therapeutic application. IT-based therapies have been more effective in hematological malignancies like lymphomas and leukemias. These diseases are normally immunosuppressive and, as a consequence, the generation of neutralizing antibodies is less frequent (73). However, in the treatment of solid tumors neutralizing antibodies are more frequently present. Several approaches are being investigated to reduce immunogenicity, e.g. the use of immunosuppressive agents or monoclonal

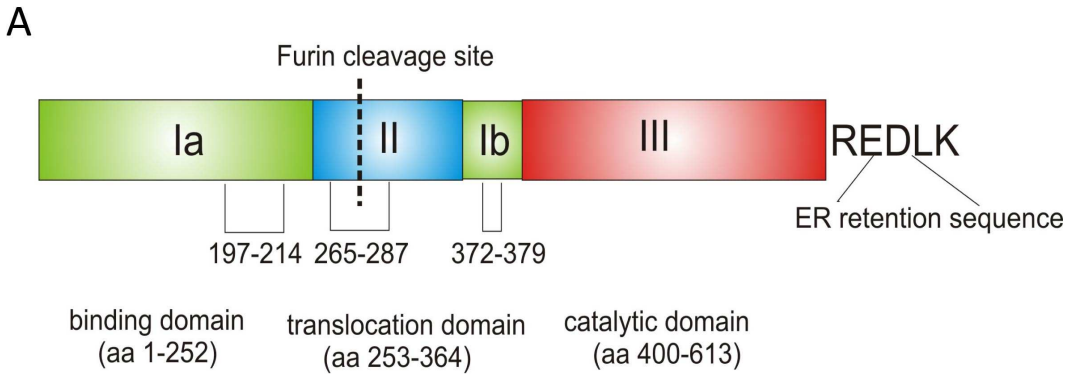
antibodies such as rituximab, which depletes normal peripheral B cells (74). Another approach is to attempt to identify the immunogenic sequences of the toxin and generate less immunogenic variants which still retain their full cytotoxic activity (75-76). The generation of toxins chemically conjugated with polyethylene glycol (PEG) (77) to shield the protein's immunoreactive sites is as well a possible alternative to overcome this problem. Probably, the combination of these approaches and others will allow repeated treatment schedules increasing the amount of the ITs at the tumor site.

Unspecific toxicity can be related to the targeting moiety when it is not specific enough for its target but cross-react with other antigens, or when the same target cancer antigen is present in high amounts on normal tissues. This can become a serious problem if these antigens are present in vital organs. Normally, this is ruled out in the extensive preclinical testing of ITs. However, it is noteworthy to mention how crucial the choice of the right TAA to be targeted and the specificity of the targeting moiety in the development of ITs are. Nevertheless, most of the unspecific effects related to ITs are associated with the toxin moiety itself. Vascular leak syndrome (VLS) and/or hepatotoxicity are frequently the dose-limiting toxicities (78). VLS is the result of endothelial cell damage increasing vascular permeability and producing a fluid leakage from capillaries. Edema, hypotension and hypoalbuminemia are signs of VLS, pulmonary edema and cardiovascular failure being the most severe consequences. Amino acid sequences that are thought to be responsible for VLS have been proposed (79) and several approaches are currently being investigated to test whether VLS can be diminished during IT treatment. Liver injury might be the result of the sensitization of hepatocytes toward TNF production by Kupffer cells after toxin activation (80), although liver toxicity has been shown in other cases to be related to the presence of the target antigen in the liver (81).

Probably new generations of ITs, targeting more specific TAAs, in combination with toxins which are modified to be less immunogenic and to produce less unspecific toxicity will further improved the application of ITs and yield new treatment options.

## *Pseudomonas* exotoxin A

*Pseudomonas* exotoxin A (ETA, PE), is one of the most frequently used toxins for the generation of ITs (82). ETA is a 66 kDa protein produced by *Pseudomonas aeruginosa* as a single polypeptide chain. It is organized in three structural and functional domains (Figure 4). The N terminal domain (domain Ia) is responsible for binding to the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2MR/LRP), a receptor present in the majority of the cells. Domain II mediates the translocation of domain III, the enzymatic domain, into the cytosol of the cells. Domain Ib is located between domain II and III and has at present no known function (83).



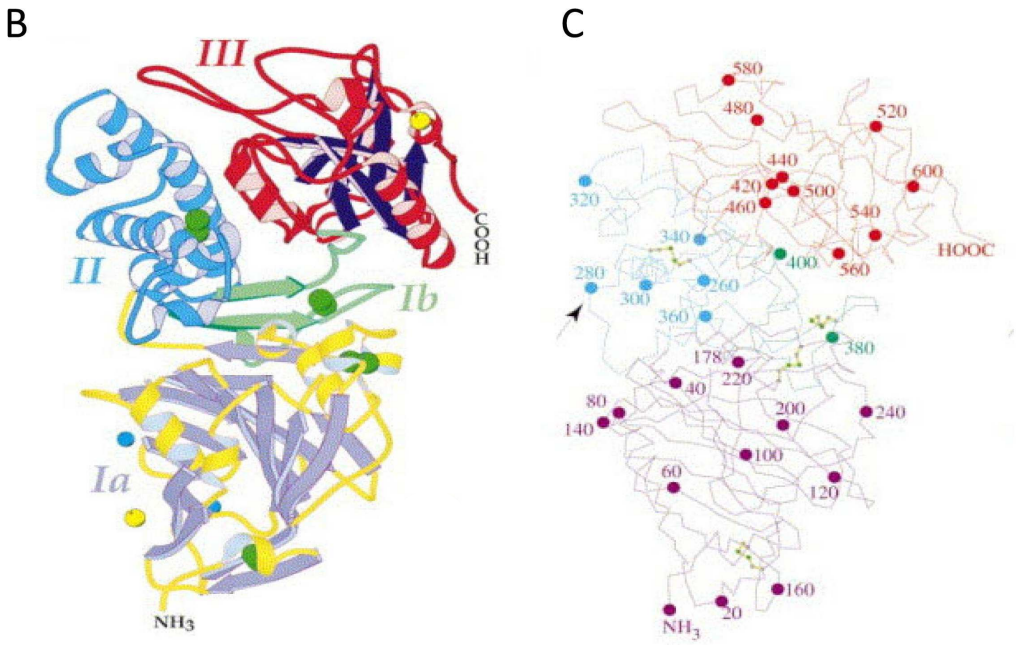



Figure 4. **Pseudomonas exotoxin A (ETA)** A) Schematic representation of ETA domains: the receptor binding domain (green), the translocation domain (blue) and the catalytic domain (red). The furin cleavage site (aa 274-280), disulfide bonds and ER retention sequence at the C-terminus are depicted. B) Three dimensional structure of ETA. domain Ia (1-252), purple b-sheet, yellow a-helices and coils; domain Ib (365-404), green b-sheet and coil; domain II (253-364), light blue b-sheet and coil; and domain III (405-613), red a-helix and coil, blue b-sheet. Cyan spheres represent Na ions; yellow spheres represent Cl ions; disulfide positions are indicated as green spheres. C) Stereographic Cα representation. Spherical main-chain atom positions are numbered every 20 amino acid residues. Color scheme and orientation based on (a). Disulfide positions are indicated as ball-and-stick side-chains. An arrow indicates the site of furin cleavage. Figure adapted from Wedeking et al.(83)

The first step of cell intoxication by ETA is its internalization by receptor-mediated endocytosis (Figure 5). Once in the endocytic compartment, it undergoes a conformational change and it is cleaved by furin, an endosomal protease (84-85). This generates two fragments which are still bound by a disulfide bridge (cys265-cys287). After reduction of this disulfide bond, a 37 kDa fragment containing the catalytic domain and an ER retrival sequence at (REDL) at its C-terminus, is transported from the early endocytic compartment to the late endosomes and to trans-Golgi network (TGN) in a Rab9 dependent manner. There, it binds to the KDEL receptor using its KDEL-like sequence, and is transported to the ER. Via this 'reverse secretory' intracellular pathway mediated by the retention sequence, ETA probably can access to the ER and translocate to the cytosol. Accordingly, analysis of several mutants demonstrated that variants with the highest affinity to the KDEL receptor showed the highest cytotoxicity (86). Alternatively, ETA bound to the α2MR/LRP receptor has been found in detergent resistant microdomains and it is likely that this fraction of the toxin can follow the direct EE to TGN pathway exploit by other lipid-binding proteins. This alternative pathway avoiding late endosomes (LE) might be cell specific depending on the amount of receptors that are recruited to DRM (87). Once the 37 kDa fragment translocates to the cytosol, it catalyzes the ADP ribosylation of the eukaryotic elongation factor -2 (eEF-2) (88). This ADP ribosylation inactivates the eEF-2 resulting in irreversible protein synthesis inhibition and cell death (Figure 5).

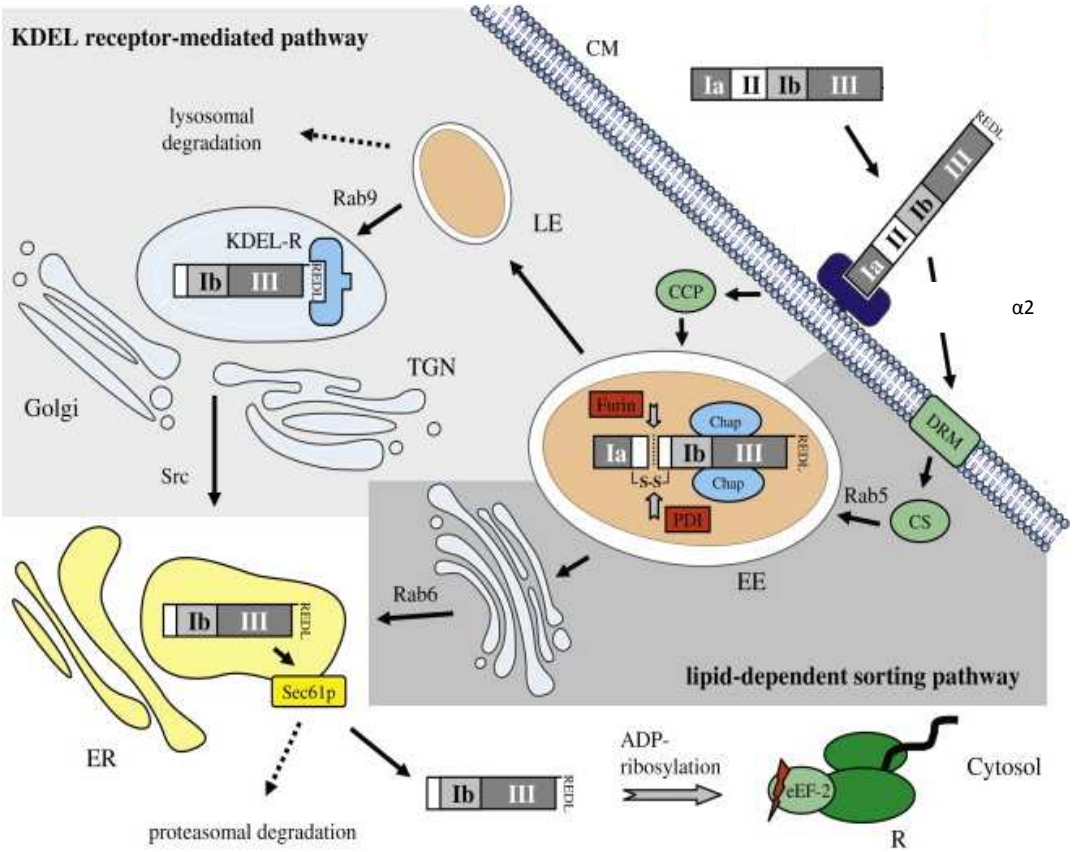



Figure 5. **Mechanism of intoxication of *Pseudomonas* exotoxin A (ETA).** After ETA binding to its receptor (α2MR/LRP), internalization occurs by clathrin coated pits (CCP) or via caveosomes (CS), in a Rab5-dependent manner, when ETA bound to the receptor is localized in detergent resistant microdomains (DRM). After processing inside of the endosomes (EE), the 37 kDa catalytic domain travels to the endoplasmatic reticulum (ER) via the trans-Golgi network (TGN) ), in a Rab9 or Rab6 dependent manner. This domain is now secreted to the cytoplasm via the translocon Sec61p, where ADP-ribosylates the elongation factor 2 (eEF-2) leading to cell death. CM: cell membrane, PDI: disuldide isomerase, Chap: chaperones, LE: late endosome. (Figure adapted from Wolf et al., (78))

## *Pseudomonas* exotoxin A-based immunotoxins

The elucidation of ETA's toxicity mechanism and of its molecular structure has allowed the generation of several immunotoxins. When domain Ia is removed from ETA to generate a 40 kDa protein, the cytotoxicity is abrogated (89) because ETA has lost its ability to bind to its cellular receptor. Taking advantage of this fact, the 40 kDa fragment can be genetically fused to specific targeting moities to redirect the toxic activity of ETA to desired target cells. Moreover, it has been demonstrated by mutants generated of a chimeric protein composed of TGFα fused to the different fragments of ETA that a portion of domain Ib can be additionally removed (aa 360 to 380) without lost in cytotoxicity (90). This generates a 38 kDa fragment which can also be used in the preparation of ITs. Although the function of domain Ib is still not clear, this is an indication that this domain is not essential for cell toxicity. It may be probably that domain Ib is required for intoxication when domain Ia is present (as they are close in the three dimensional structure of ETA). It might also be possible that domain Ib is necessary for secretion of ETA from P*seudomonas aeruginosa*. The replacement of ETA carboxy-terminal sequence, REDLK, by the ER retrieval sequence KDEL, enhances ETA toxicity (86, 91).

Several ETA-based ITs are being evaluated in preclinical trials and some have entered into clinical trials for the treatment of various cancers (78). Some examples are briefly described.

Promising IT clinical data have been obtained using BL-22 for the treatment of hairy cell leukemia (92). BL-22 (RFB4 dsFv-PE38) consists of a dsFv directed to the CD22 antigen fused to the truncated form of ETA, PE38. BL-22 has been investigated in phase I with complete remission in the majority of the patients. A phase II trial has been completed which has confirmed the phase I results. In addition, a high-affinity mutant of BL22 (HA22) is in phase I testing (93).

LMB2 (Anti-Tac (Fv)-PE38KDEL) targets the alpha subunit of IL-2R and contains PE38 as the toxin moiety. LMB-2 was given to patient with chemotherapy-resistant leukaemia, lymphoma and Hodgkin disease in a phase I trial. It was the first IT that showed benefit for patients with hairy-cell leukaemia (94).

4D5MOCBETA (VB4-845) is another promising ITs for the treatment of solid tumors. It is composed of a humanized scFv fragment (4D5MOCB) specific for EpCAM genetically fused to a truncated form of ETA (58). Two formulations of this IT have entered clinical trials under the trade names of Proxinium and Vicinium (Viventia Biotech). Proxinium is used for the treatment of head and neck cancer and Vicinium is used for the treatment of bladder cancer. Phase I clinical trials have been completed with Proxinium and to further evaluated the safety and efficacy Phase II and III studies have been started. Vicinium has completed phase I clinical trials and is undergoing a phase II clinical trial. (57)

Several ITs that target the Lewis Y antigen, which is highly expressed in many epithelial tumors, have also been tested in the clinic (95-96). Mesothelin is another TAA which is targeted by ITs, due to its overexpression on mesothelioma, ovarian and pancreatic cancer (97).

Except for some cases (98-99) ETA-based ITs have been mainly accomplished with antibody fragments such as scFv and dsFv. For this reason they have to be expressed in the periplasm of bacteria or need to be refolded after expression in inclusion bodies. These methods result in low protein yields or laborious refolding and purification procedures to obtain biologically active proteins after renaturation. Since for ITs no other feature of the antibody is required than its antigen binding ability, a solution might come from the use of alternative non-IgG binding scaffolds as targeting moieties ((100) and Martin Killias et al., in preparation). These can be engineered to have the same or even better specificity and affinity and to improve production yield and stability.

# Alternative binding molecules

MAbs offer many attractive features for tumor targeting, as mentioned before. However, they suffer from clear limitations especially when merely used to deliver a payload: High manufacturing costs, a complex molecular format, laborious production and limited efficacy due to their rather large size and low tissue penetration. To overcome some of these limitations several antibody fragments have been generated such as Fab, scFv and dsFv (101). Although promising, they are still based on the immunoglobulin format and consequently they share some of the antibody's sub-optimal biophysical properties. Over the past years several strategies based on different protein topologies than the immunoglobulin format have been developed. Ideally, these alternative binding molecules should overcome the shortcomings of antibodies while keeping the target affinity and specificity (102)

Numerous alternative binding proteins have been characterized and their application in research, diagnostics and therapy are being analyzed (103-104). Of these, Designed Ankyrin Repeat Proteins (DARPins) form a particularly promising innovative approach.

## Ankyrin repeat proteins and DARPins

Ankyrin repeat (AR) proteins are natural binding molecules which are involved in diverse cellular functions. They have been found across all phyla ranging from viruses to humans. The ankyrin repeat has been found in more than 400 proteins such as cell cycle regulators, cytoskeletal organizers and tumor suppressors (105-106). These proteins are present in nucleus and cytoplasm indicating that they can adapt to different environments. Ankyrin repeat domains (AR) are repeating structural units of 33 amino acids, which stack

together to form elongated proteins (107). Structurally, each repeat consists of a beta turn followed by two antiparallel alpha helices and a loop reaching the turn of the next repeat. The number of repeats within one protein is variable.

To take advantage of the properties of ankyrin repeat proteins as binding molecules, a library was engineered by consensus design (108). In this approach six of the 33 amino acids in each consensus repeat were randomized and libraries containing 2 or 3 repeats were generated. To shield the hydrophobic core a capping repeat was added at each the N and C terminus (Figure 6). Members of the libraries are named Designed Ankyrin Repeat Proteins (DARPins). This strategy produced not only large libraries but also DARPins with optimal biophysical properties (109). These libraries are a valuable source of binding molecules, which was shown by selection of high affinity binders against several types of proteins (110-113). The simple molecular and modular architecture of DARPins allows a broad range of applications. This, taken together with their high affinity and selectivity makes DARPins attractive candidates for further drug development.

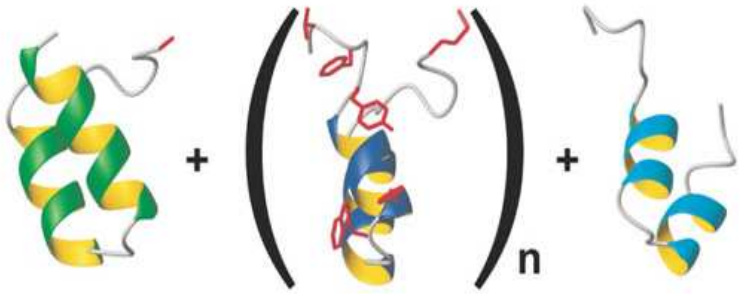



Figure 6. **Schematic representation of the DARPin library** (114). In green the N-terminal capping repeat, follow by a variable number of internal repeats in blue and in cyan the C-terminal capping repeat. Randomized amino acid side chains are depicted in red.

## DARPins in targeted cancer therapies

DARPins have been selected against several TAAs such as HER2 (113, 115), EGFR, ErbB4 (116) and EpCAM (Martin Killias et al., in preparation) using both ribosome display and phage display. Due to their high affinity, specificity, easy production process, small size and robustness, they can be consider as ideal molecules for targeted therapies. Several molecular formats might be possible using DARPins (117) which opens the door to different therapeutic applications. DARPins can be employed as monovalent or bivalent binders, in a bispecific construct, or conjugated to various effector molecules. As examples, Her2-specific DARPins were successfully used in situ for staining of sections of breast carcinoma (115), and bivalent Her2-DARPins are currently being investigated as potential therapeutic agents with very promising results *in vitro* (Tamaskovic, personal communication). In addition, EGFR-specific DARPins were able to inhibit cell proliferation of EGFR-positive cells (Boersma et al., in preparation). EpCAM-specific DARPins fused to protamin were successfully used to deliver siRNA to tumor cells (118) and a EpCAM-DARPin fused to ETA have shown potent antitumor activity on tumor xenografts (Martin Killias et al, in preparation). All these data warrant the further development of DARPins toward future clinical applications.

# Aim of the thesis

This thesis describes the generation of a novel system for the delivery of a truncated form of Pseudomonas exotoxin A (ETA") to tumor cells. An EpCAM-specific DARPin, fused to the toxin, is used as the targeting moiety. The rationale for this approach is to take advantage of the favorable characteristics of DARPins as a targeting moiety in conjunction with the favorable properties of EpCAM as a tumor marker.

The first goal was to select DARPins specifically recognizing EpCAM with high affinity. To this purpose, the extracellular domain of EpCAM was produced, purified and used as a target for selection. DARPins were selected with a novel phage display system, SRP phage display, a system adapted for highly stable and fast folding proteins. As an alternative approach, ribosome display and affinity maturation procedures were performed. Using both selection systems, several EpCAM-specific DARPins were selected and characterized.

The second part of the project was to asses the potential of EpCAM-specific DARPins for tumor targeting and delivery of ETA". To this end, one of the characterized DARPins, Ec4, was genetically fused to ETA" to generate the first EpCAM-specific DARPin-toxin, Ec4-ETA". This DARPin-toxin fusion protein was biochemically characterized and its cytotoxicity and antitumor activity were evaluated both *in vitro* and *in vivo.*

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**Chapter 2**

# DARPins recognizing the tumor associated antigen EpCAM selected by phage and ribosome display

**Article in preparation**

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# DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display

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Running title: Selection of EpCAM-specific DARPins

## Abstract

Designed Ankyrin Repeat Proteins (DARPins) represent a novel class of binding molecules. Their very favorable biophysical properties such as high affinity, stability and expression yields make them ideal candidates for tumor targeting. Here, we describe the selection of DARPins specific for the tumor-associated antigen epithelial cell adhesion molecule (EpCAM), a relevant target for cancer therapies. Several DARPins were selected from combinatorial libraries by phage display and ribosome display. The first generation of EPCAM binders was then further improved. The resulting second generation of DARPins were expressed at high yields and specifically bound to EpCAM-expressing cells. One of the binders, denoted Ec4, bound to EpCAM with picomolar affinity (Kd=917.2 ± 77.8 pM). In addition, it was internalized upon receptor binding, an important prerequisite for delivery of cytotoxic agents to the tumor. Taken together, the results indicate that EpCAM-specific DARPins could be of key interest as an alternative to antibodies for tumor targeting.

## Introduction

The epithelial cell adhesion molecule (EpCAM) is a tumor-associated antigen which is overexpressed in many solid tumors (1-3) and was recently identified as a cancer stem cell marker (4-5). Whereas EpCAM was initially considered to be a homophilic cell-cell adhesion molecule, more recent studies demonstrated its role as an oncogenic signaling molecule, which is activated via regulated intramembrane proteolysis, with the cleaved cytoplasmic domain driving the expression of the proto-oncogen c-*myc* and the cell cycle regulators *cyclin A* and *cyclin E* (6-7). Moreover, there is evidence that EpCAM interacts with a number of metastasis associated proteins in the cell membrane, such us CD44, tetraspanins and claudin 7 (8-9).

The high level of EpCAM in solid tumors and its limited expression in normal epithelial tissues (10), whose basolateral expression moreover render it poorly accessible, makes it attractive for tumor-targeting (11-13). In previous work we generated an EpCAM-specific immunotoxin from a stability engineered anti-EpCAM scFv fused to truncated *Pseudomonas* exotoxin A (14), which is currently in phase II clinical investigation for the treatment of non-invasive bladder cancer (15). In addition, we have developed EpCAM-targeted nanoscale delivery systems which were loaded with therapeutic antisense oligonucleotides or doxorubicin, and which were tumor-targeted by the same scFv antibody (16-17). Since the production process for the recombinant antibody fragments, especially in conjunction with drug delivery systems is still demanding, alternative binding molecules, such as more stable and better expressing non-IgG scaffold proteins might be advantageous (18). This will especially allow to construct formats which are not accessible at all for scFv derived constructs.

Designed Ankyrin Repeat Proteins (DARPins) are well-suited for this purpose, since they show favorable biophysical properties, such as high expression yield and low aggregation tendency (19-22). Their stable and flexible structure, small molecular weight (14 to 21 KDa) and ease of production make them suitable tools for biomedical applications. Moreover, the absence of cysteines allows site-specific modification and conjugation with various effector moieties. The ankyrin repeat is built from two antiparallel helices and a loop extending in perpendicular direction, and potential interaction residues were

randomized, while residues important for maintaining the repeat structure (framework) were defined and kept constant in a consensus design approach. DARPin libraries with different numbers of repeats (two or three) were assembled in between N- and C-terminal capping repeats, shielding the hydrophobic core (19). Recently, the C-terminal repeat was redesigned to further improve the stability of these proteins (23).

High affinity binders against several target proteins have been isolated from DARPin libraries mostly using ribosome display (20-22). Ribosome display is performed entirely *in vitro* (24-25) and in combination with error-prone PCR constitutes an ideal tool for affinity maturation of binding proteins, since mutations can be easily introduced after each selection round (26). Phage display was also used to select DARPins from libraries, but the standard filamentous phage system had to be modified for the display of these proteins rapidly folding already in the cytoplasm. By using a signal sequence targeting the signal recognition particle (SRP) dependent pathway (27), co-translational transport is achieved, and such a DARPin phage library was successfully used to efficiently select binders against a wide range of targets (28).

Binding proteins destined for therapeutic applications need to fulfill many stringent criteria, and a wide range of targeted epitopes is thus useful initially. It was therefore of interest to select with ribosome display and phage display in parallel. Indeed we found different binders from both methods, which we can partially rationalize. Moreover, we could by subsequent evolutionary rounds of ribosome display not only improve affinities to the picomolar range but also improve the biophysical properties of the evolved binders. We characterized the evolved binders on cells and some DARPins were analyzed for internalization upon binding as an important prerequisite for efficient delivery of cytotoxic compounds to target cells.

## Material and Methods

### Cell lines and culture condition.

The colorectal carcinoma cell line HT29, the breast adenocarcinoma cell line MCF7, the non-Hodgkin's lymphoma cell line RL, the squamous cell carcinoma cell line of the tongue CAL27, the prostate carcinoma cell line LNCAP and the human embryonic kidney cells HEK293T were obtained from ATCC (American Type Culture Collection). The small cell lung carcinoma cell line SW2 was maintained in our laboratory for many years. All cell lines were grown in DMEM (Dulbeccos's modified Eagle's medium) (Sigma, Buchs, Switzerland). Culture medium was supplemented with 10% heat inactivated fetal bovine serum (Amimed, Bioconcept, Allschwil, Switzerland), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. All cells were tested negative for mycoplasma using MycoAlert (Lonza, Basel, Switzerland ).

### EpCAM expression and purification.

The cDNA encoding EpCAM was kindly provided by Dr. M. Münz (Micromet AG, Munich, Germany). The extracellular domain of EpCAM (EpEx), residues 1 to 265 (including the signal sequence), was cloned into the plasmid pCDNA3.1(-) for mammalian cell expression. Two additional tags were added to the C-terminus of the protein, an Avi-tag (GLNDIFEAQKIEWHE) for biotinylation followed by a $His_6$ tag for protein detection and purification. The expression vector was transiently transfected into HEK293T cells using the standard calcium precipitation protocol. Four days after transfection, cell culture media was collected, centrifuged and filtrated to eliminate cell debris.

The supernatant containing EpEx was concentrated and loaded on a Ni-NTA column packed with Ni-NTA superflow (Qiagen, Hilden, Germany). After washing with 5 column volumes (50 mM $NaH_2PO_4$ pH 8.0, 500 mM NaCl, 10% glycerol and 10 mM imidazole), EpEx was eluted with elution buffer (50 mM $NaH_2PO_4$, pH 8.0, 300 mM NaCl, 10% glycerol and 250 mM imidazole). After purification, EpEx was dialyzed against 10 mM tris and enzymatically biotinylated. One mg of protein (at a concentration of 1 mg/ml) was incubated

with 10 µg of BirA (Avidity, Aurora/Colorado, USA) at room temperature overnight in a buffer containing 50 mM bicine pH 8.3, 10 mM ATP, 10 mM $Mg(CH_3COO^-)_2$ and 40 µM biotin. Biotinylated EpEx (bEpEx) was dialyzed against PBS (15 mM $KH_2PO_4$, pH 7.4, 81 mM $Na_2HPO_4$, 27 nM KCl, 137 mM NaCl) to remove unreacted biotin. Efficient biotinylation was confirmed by using ELISA and western blotting and detection with a streptavidin- horseradish peroxidase conjugate as detection reagent (Roche, Basel, Switzerland).

**Selection of DARPins by SRP phage display and epitope masking**

The selection of EpCAM-specific DARPins by phage display was performed essentially as previously described (29). For the first selection round on immobilized target protein, 66 nM neutravidin (Pierce, Rockford, USA) was coated on MaxiSorp immunotubes (Nunc, Roskilde, Denmark). Tubes were blocked with PBSTB (PBS, 0.1% Tween-20, 0.2% BSA) for 1 h and bEpEx (1 ml, 400 nM) was added for 2 h at 4°C. The second and third rounds were performed on MaxiSorp plates (Nunc). Neutravidin (Pierce) or streptavidin (Sigma) were coated to the plates (66 nM, 100 µl/well in PBS) at 4°C overnight. After washing, bEpEx (100 nM, 100 µl/well) was added for 1 h at 4°C. To avoid selection of binders against neutravidin or streptavidin, these two proteins were used alternately in subsequent rounds. At the end of the third round, enrichment of specific binders was monitored by phage ELISA.

For epitope masking, Eph1 was expressed in *E. coli* and purified by IMAC as described below (purification of DARPins). Pools of phage particles after the first round on immobilized protein (in tubes) were used as input material. bEpEx (100 nM) was incubated with EPh1 (10 µM) for 1 h before a standard selection round on soluble protein was performed, where complexes of phages bound to bEpEx were captured with streptavidin-coated paramagnetic beads (Dybabeads MyOne Streptavidin T1, Dynal, Invitrogen, San Diego, USA) for 20 min. After three rounds of selection with epitope masking, phage enrichment was analyzed by phage ELISA in the presence or in the absence of an excess of EPh1.

**Phage ELISA**

Phage ELISA was performed to detect specific enrichment of phages against EpCAM. Neutravidin (66 nM), Streptavidin (66 nM) and human IgG1 Fc domain (100 nM) were directly coated to MaxiSorp plates (Nunc) overnight at 4°C. Biotinylated proteins, bEpEx (100 nM) and ErbB4 (100 nM), were coated via neutravidin for one hour at 4°C. Output phages from each round (~$1 \cdot 10^5$ phages) were incubated with the target and control proteins for 2 h at room temperature. After 3 washes with PBST (PBS, 0.1% Tween-20), bound phages were detected with mouse anti-M13 antibody horseradish peroxidase conjugate (Glattbrugg/Zürich, Switzerland).

**Selection of DARPins by ribosome display**

Both N2C and N3C DARPin libraries (19) were used to select for DARPins binding to EpEx. Three rounds of selection by ribosome display were performed essentially as described before (25). Briefly, for each round the translation mix containing mRNA-ribosome-DARPin complexes was incubated for 1 h at 4°C with 100 nM bEpEx in solution. The complexes bound to EpCAM were captured incubating with 100 µl streptavidin-coated paramagnetic beads (Dynabeads MyOne Streptavidin) for 30 min at 4°C. After washing the beads with WBT (50 mM Tris acetate, pH 7.6, 150 mM NaCl , 50 mM $Mg(CH_3COO^-)_2$, 0.01% Tween-20), the mRNA was eluted with elution buffer (50 mM Tris acetate, pH 7.6, 150 mM NaCl, 250 mM EDTA) and prepared for another round of selection. To minimize selection of unspecific binders, all tubes were pretreated with TBST (50 mM Tris acetate, pH 7.6, 150 mM NaCl, 0.05% Tween-20) supplemented with 0.1% of BSA. To further avoid binders against the streptavidin present on the paramagnetic beads, before each panning step, the translational mix was preincubated with the beads for 1 h at 4°C.

**Affinity maturation**

Selection of second generation binders was performed as described (25-26) with a few modifications. First, EpCAM-specific DARPins selected by phage display or ribosome display, were randomly mutated by error-prone PCR. Each DARPin was amplified individually in the presence of 3, 9 and 20 µM dNTP

analogues dPTP (6-(deoxy-β-D-erythro-pentofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazine-7-one-5'-triphosphate) and 8-oxo-dGTP (8-oxo-2'-deoxyguanosine-5'-triphosphate). Each amplification was performed in a 50 µl reaction in the presence of 1 µM primers, one unit of Platinum*Taq* DNA Polymerase (Invitrogen), 5% DMSO, 1.5 mM $MgCl_2$ and 200 µM dNTPs. PCR products were then pooled in equimolar amounts for selection experiments.

Four rounds of ribosome display were performed. In rounds 1 and 3, the translation mix was incubated for 1 h at 4°C with 0.7 µM bEpEx and subsequently a 5000-fold excess of non-biotinylated EpEx was added (30). This mixture was kept under slow shaking at 4°C for 2 or 10 h, respectively, to select for binders with a slow off-rate. In rounds 2 and 4, 100 nM bEpEx were used in the panning step and no non-biotinylated competitor was added to enrich correct clones without further selection pressure. mRNAs of complexes bound to bEpEx were isolated as described above.

**Purification of DARPins**

Selected DARPins from phage and ribosome display were cloned into a pQE30-derived vector (Qiagen, Hilden, Germany), containing an N-terminal MRGS$(H)_6$ tag and a C-terminal double stop codon (pQE30ss) or into pQE30_sfGFP, the latter to create DARPins C-terminally fused to superfolder GFP (31). For ELISAs, affinity measurements, labeling and flow cytometry DARPins were produced in soluble form in *E. coli* XL1-blue (Stratagene, La Jolla, USA) and purified using Ni-NTA Superflow (Qiagen) in gravity flow columns as described (19). For SPR measurements, the proteins were further purified by preparative size exclusion chromatography.

**Size Exclusion Chromatography**

DARPins (15 µM) in 50 µL PBS were analyzed on a Superdex-200 PC 3.2/30 column using either an ETTAN or ÄKTAmicro chromatography system (all GE Healthcare) at a flow rate of 60 µl/min and with PBS as running buffer. For preparative size exclusion chromatography, DARPins were purified on a Superdex-200 10/300 GL column using an ÄKTAexplorer chromatography system (GE Healthcare) at a flow rate of 1 ml/min.

**ELISAs**

MaxiSorp plates were coated with 100 µl of 66 nM neutravidin in PBS overnight at 4°C. After washing, all wells were blocked with 300 µl PBS containing 0.2 % of bovine serum albumin (BSA) for 1 h at room temperature. Protein targets were biotinylated and added to the wells to a final concentration of 50 nM (10 nM in competition ELISAs). Crude bacterial extract (100 µl, for screening of single clones from phage display and ribosome display selections) or purified DARPins (100 µl, 200 nM for specificity and 10 nM in competition ELISAs) were added at room temperature. After 1 h, bound DARPins were detected with an anti RGS-$His_6$ antibody conjugated with horseradish peroxidase (Qiagen) or with an anti-RGS-$His_6$ antibody (Qiagen) and an anti-mouse-IgG conjugated to alkaline phosphatase (Pierce). For the competition ELISA, the same setup as described above was used except that purified DARPins were incubated with 100 nM non-biotinylated EpEx prior to incubation with immobilized EpCAM for 10 min at room temperature.

**Labeling of DARPins with AlexaFluor-488**

EpCAM-specific DARPins as well as control DARPins were cloned into a pQE30-based vector, appending two glycines and a cysteine. These DARPins containing a unique cysteine at their C-terminus were expressed and purified as described above. Cys-DARPins were fully reduced by incubation with 100-fold excess of tris-(2-carboxyethyl)phosphine (TCEP) for 30 min at 37°C in PBS pH 7.4. TCEP removal and buffer exchange to degassed PBS pH 7.1 was achieved with a HiTrap desalting column (GE Healthcare). AlexaFluor488-C5-maleimide (Invitrogen) was added in 2-fold excess and incubated at 25°C for 2 h. After quenching unreacted dye with 10 mM dithiothreitol (DTT) for 20 min at 25°C, a PD-10 column (GE Healthcare) was used to remove unreacted dye and exchange the buffer to 100 nM sodium-bicarbonate with 20 nM NaCl, pH 8. Monolabeled DARPin was separated from unlabeled protein by anion exchange chromatography on a MonoQ 5/50 GL column (GE Healthcare) using 100 nM sodium bicarbonate with 1 M NaCl at pH 8 for isocratic elution.

**Flow cytometry analysis.**

Flow cytometry analysis was performed to confirm the binding of DARPins to EpCAM expressed on the surface of a variety of tumor cell lines. $1 \cdot 10^6$ cells were harvested and washed two times with PBS, then resuspended in FACS buffer (PBS, 1% BSA) containing the DARPin (fused to GFP, coupled to Alexa488 or unlabeled) (100 nM) and incubated for 45 minutes on ice. Unlabeled DARPins were detected using anti-RGS-His antibody (Qiagen) and a goat anti-mouse FITC labeled secondary antibody (Invitrogen). Cells were washed three times between all incubation steps with FACS buffer. After the last wash, cell were resuspended in 1 ml FACS buffer and subjected to flow cytometry using a FACSCalibur or FACSCanto II flow cytometer (both BD Bioscience). For each experiment 10,000 cells were counted. Data were analyzed using the FlowJo software (Tree Star, Ashland/Oregon, USA)

For competition experiments with unlabeled DARPins, 500,000 HT29 cells were incubated with 100 nM Ec4-sfGFP or Ac2-sfGFP fusion proteins or the monoclonal anti-EpCAM antibody MOC-31 for 30 min on ice. For competition, the cells were preincubated with 10 µM DARPins Ec4 or Ac2 for 15 min on ice. MOC-31 treated samples were additionally incubated with 2 µg/ml Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen) for 20 min on ice. Cells were then analyzed by flow cytometry as described above.

**Affinity determination**

*Affinity determination on cells.* Harvested MCF-7 cells were pre-incubated for 30 min at 37°C in FACS buffer supplemented with 0.2% sodium azide to inhibit internalization. For equilibrium experiments, $3 \cdot 10^5$ cells in 500 µl were incubated for 1 h at 4°C with different concentrations of DARPin-sfGFP ranging from 100 pM to 200 nM. After one washing step, mean fluorescence intensities (MFIs) were measured on a FACSCanto II flow cytometer (BD Bioscience). For dissociation rate experiments, $3 \cdot 10^5$ cells were saturated for 1 h at 4°C with 100 nM DARPin-sfGFP. Then, the cells were centrifuged (300 g, 3 min, 4°C) and resuspended with 1 µM DARPin as an unlabeled competitor to prevent rebinding. MFIs were recorded at different time points between 0 to 4 h. For association rate experiments, $3 \cdot 10^5$ cells were incubated with 2.5, 7.5 or 22.5 nM DARPin-sfGFP and measured at times between 1 and 60 min without prior

washing. Background association of an unselected library member E3_5 fused to sfGFP was subtracted. Data evaluation was performed with Prism (GraphPad).

*Affinity determination with surface plasmon resonance.* All measurements were performed on a Biacore 3000 instrument. A streptavidin (SA) chip was prepared according to the manufacturer's protocol and was coated with bEpEx to about 120 resonance units. DARPins ranging from 1 to 100 nM were injected at a buffer flow of 30 µl/min in HBST (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.05% Tween-20). Data evaluation was performed using BIAEVAL (Biacore) or Scrubber 2 (Biologic Software).

**Internalization by Confocal microscopy**

MCF-7 cells ($2 \cdot 10^5$ cells) were seeded on coverslips and incubated for 24 h at 37°C and 5% $CO_2$. EpCAM-specific DARPins, EPh1, and the unspecific DARPin off7 labeled at an engineered C-terminal cysteine (see above) with AlexaFluor-488 (AlexaF488) were added to the cells at a final concentration of 100 nM (diluted in DMEM). After 1 hour of incubation at 37°C or at 4°C with the labeled DARPins the cells were washed three times with PBS for 5 minutes each time and fixed with 4% paraformaldehyde in PBS (10 minutes at room temperature). Thereafter the cells were rinsed with PBS and the nuclei were stained with 0.8 µg/ml 4',6-diamidino 2-phenylindole (DAPI) in PBS for 2 min. The coverslips were then quickly washed with PBS, mounted in glass slides with Fluoromount G (Southern Biotech) and sealed with nail polish. The glass slides were kept in the dark at 4°C until confocal microscopy was done. Fluorescent images were recorded using a confocal laser scanning microscope (TCS-SP 2, Leica Mannheim, Germany) with a selected confocal plane approximately in the middle of the cell. Images were processed using Imaris 3D software (Bitplane).

# Results

## Selection of EpCAM-specific DARPins using SRP phage display

To maximize the chances of covering different epitopes on EpCAM, DARPins were selected both with SRP phage display (27-28) and ribosome display (see below). Three rounds of SRP phage selection were performed using the biotinylated extracellular domain of human EpCAM (bEpEx) immobilized to, alternately, neutravidin or streptavidin, itself bound to a solid plastic surface. The phage display library is in the N3C format. Specific EpCAM enrichment was already observed in the second round (Figure 1A), but it was notably stronger after the third round. Individual DARPins selected in the third round were screened for specific EpCAM binding by crude extract ELISA. DNA sequencing of 20 clones revealed one dominant clone, named EPh1. This binder contained a deletion of two amino acids between the N-terminal capping repeat and the first internal repeat. This deletion seemed to be important for EpCAM binding, since mutants were the deletion was corrected, showed reduced affinity to EpCAM when tested by ELISA (data not shown). *Epitope masking*. To increase the diversity and select DARPins binding to different EpCAM epitopes, we used an epitope masking strategy (32). The dominant binder obtained by the initial selection step, EPh1, was expressed, purified and added to new selection rounds in 100-fold excess to block the dominant epitope and to focus the selection against other epitopes. Since immobilization of the antigen can mask potential binding epitopes, the new selection rounds were performed in solution. The output from the first selection round on immobilized target formed the starting material for another four rounds of panning in solution. Output phages from round 3em and 4em (em: epitope masking) were analyzed by phage ELISA for binding to EpCAM in the presence and absence of the dominant binder EPh1 (Figure 1B) suggesting that some of the newly selected DARPins recognized different epitopes than EPh1. Individual DARPins from round 4em were analyzed by crude extract ELISA (Figure 1C) and 20 binders were sequenced. The results revealed 5 new EpCAM binders, termed EPh2 to Eph6

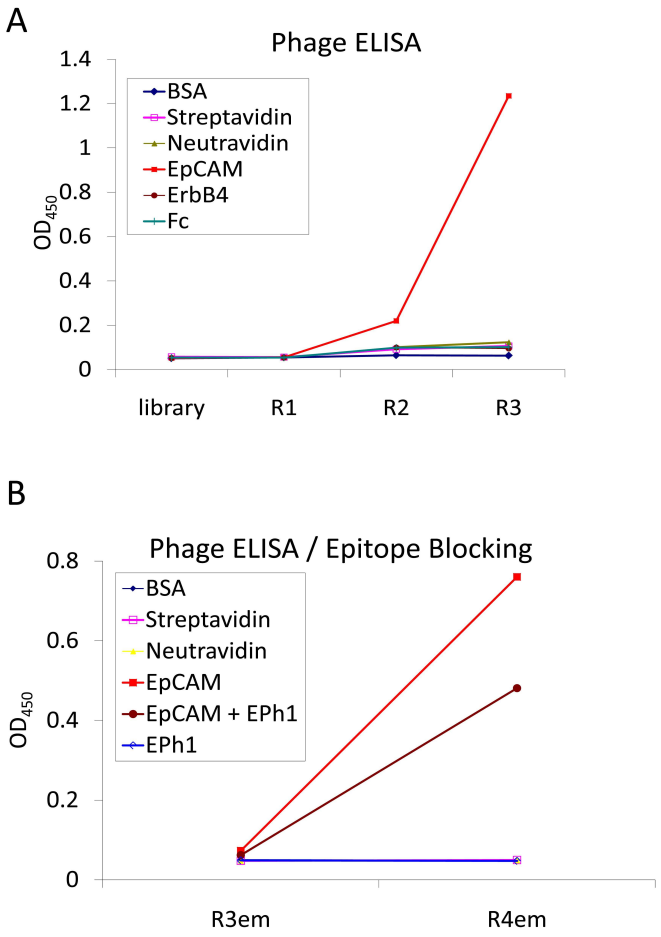



**Figure 1. Phage display selection. A) Enrichment of EpCAM binders by phage ELISA.** BSA, Streptavidin, Neutravidin, EpCAM, ErbB4 or human IgG1 Fc were immobilized on a plate and equal amounts of the initial phage library or of the output phages after each round (R1, R2 and R3) were added to the plate. The bound phages were detected using an anti M13 antibody horseradish peroxidase (HRP) conjugate. **B) Enrichment of EpCAM binders after epitope masking by phage ELISA.** As describe in fig 1A, specific enrichment of phages from round 3em and 4em (epitope masking) was analyzed. In addition, enrichment of phages against EpCAM was tested in the presence of an excess of the blocking agent EPh1 (EpCAM + EPh1). Biotinylated EPh1 was coated to the plates to test for enrichment against the blocking agent.

C

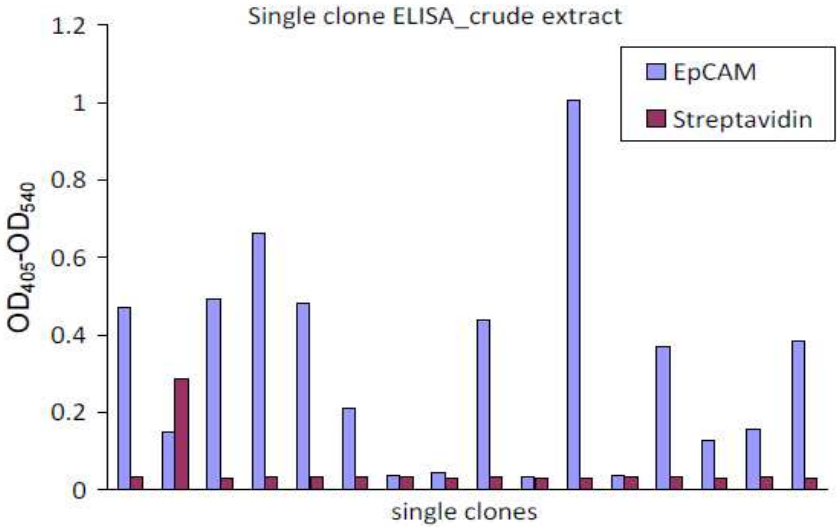



**Figure 1 C) ELISA screening of single clones.** *E.coli* extracts of randomly picked clones expressing DARPins of selection round 4em (epitope masking) were analyzed for specific binding to EpCAM and to streptavidin.

## Expression, purification and specificity of the selected DARPins

The selected DARPins, including EPh1 with the small deletion, were expressed at high levels in soluble form in the cytoplasm of *E. coli* XL1-Blue (up to 140 mg/L). All proteins were purified by a single immobilized metal affinity chromatography (IMAC) purification step (Figure 2A). The specificity of the selected DARPins was determined by ELISA (Figure 2B). E3_5 (33), an unselected member of the DARPin library, served as a control and showed no binding to EpCAM or any of the control proteins.

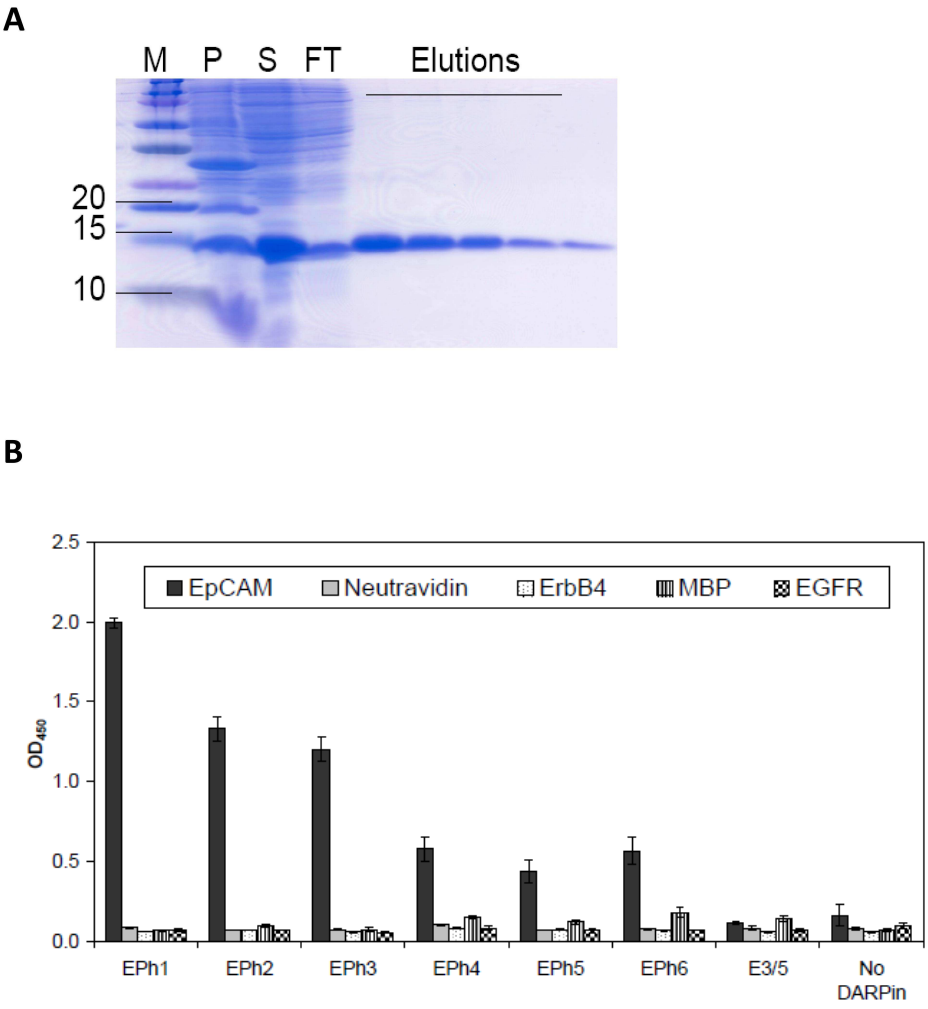



**Figure 2**. **A) SDS-PAGE analysis of DARPin purification.** A representative EpCAM-specific binder was purified by one-step Immobilized Metal Affinity Chromatography (IMAC). Fractions from purification were visualized by Coomassie blue. Lanes: M, molecular weight marker (indicated in kDa); P, bacterial pellet; S, supernatant from cell lysate; FT, Flow-through from IMAC column; Elutions, eluted fractions from IMAC. **B) Binding specificities of selected DARPins.** The 6 selected DARPins were purified and tested in ELISA for binding to EpCAM, neutravidin, ErbB4, MBP (maltose binding protein) and to EGFR (epidermal growth factor receptor). As further controls, an unselected member of the library (E3/5) and the background binding of the detection antibodies are shown in the absence of any DARPin (No DARPin).

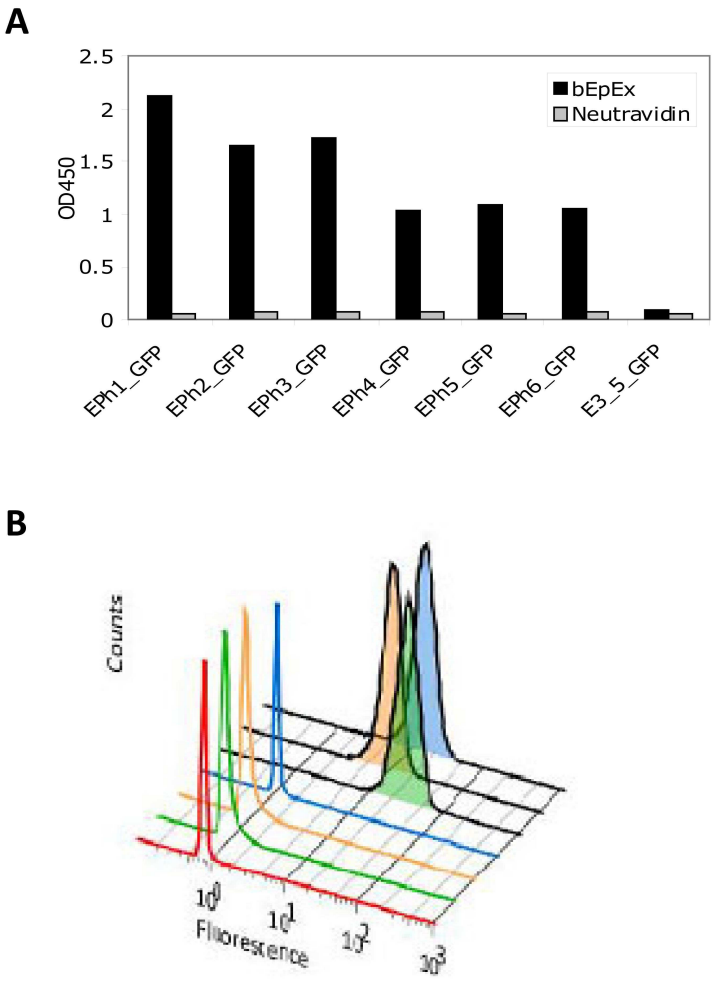
## EpCAM binding of the selected DARPins on the cells and EPh1 epitope characterization.

We then investigated whether the selected binders were able to bind to the antigen on the cell surface as well. To this end, the DARPins were genetically fused to superfolder green fluorescent protein (sfGFP)(31), and analyzed for binding to the EpCAM-positive MCF-7 (breast adenocarcinoma) tumor cell line using flow cytometry. DARPin_sfGFP fusion proteins expressed very well and showed EpCAM-specific binding in ELISA (Figure 3A) and maintain their fluorescence. For antibody fragments, such direct GFP fusions can only be produced with very low yields (34). To assess whether GFP would interfere with or unspecifically contribute to binding of the DARPins to EpCAM on the surface of viable cells, we used two additional detection methods: (i) unfused DARPins were incubated with target cells and detected with a primary (anti His-tag, located at the N-terminus) and a secondary FITC-labeled antibody using flow cytometry and (ii) EpCAM binders were cloned in a vector containing a C-terminal AviTag for in vivo biotinylation. The biotinylated binders were expressed, purified and incubated with streptavidin Alexa Fluor 488.

Flow cytometry indicated the same result for all three detection methods (Figure 3B): while all selected DARPins showed specific binding to EpCAM in ELISA, only one, EPh1, was able to bind to EpCAM in its native conformation on the cell surface of the MCF-7 cells. This was observed in all three approaches. This indicates the validity of the measurements with DARPin-GFP fusions. DARPins that did not bind to native EpCAM on the cell membrane were not further studied. Their appearance suggests that epitopes were exposed by the purified EpCAM which are hidden in the protein on the cell surface.

To confirm that EPh1 binds to EpCAM on various tumor cell types and thus would be suited for EpCAM-specific tumor targeting, we investigated its binding to four other EpCAM-positive cell lines of different histological origin (CAL 27 tongue squamous cell carcinoma, SW2 small cell lung cancer, LNCAP prostate carcinoma and HT29 colorectal adenocarcinoma) using flow cytometry. As shown in Figure 3C, EPh1 bound to all EpCAM-positive cell lines, while no binding was measured on the EpCAM-negative cell line RL (non-Hodgkin's lymphoma).

We compared the EPh1 epitope with that of the well-characterized monoclonal antibody Moc31, which recognizes an epitope present on the extracellular EGF-like domain of EpCAM (35). EPh1-specific binding to EpCAM was reduced in the presence of Moc31 in both ELISA and flow cytometry (Figure 3D), indicating that EPh1 and Moc31 bind to an at least partially overlapping epitope. As a further control, it was shown that soluble EpEx could compete the specific binding to immobilized bEpEx in ELISA.



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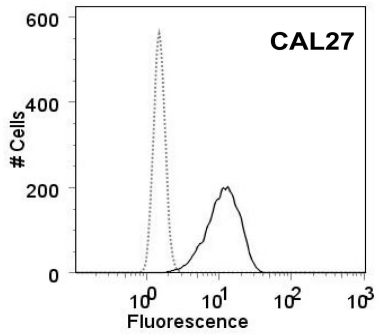
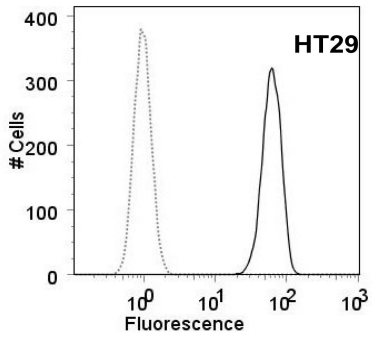

CAL27
# Cells
Fluorescence


HT29
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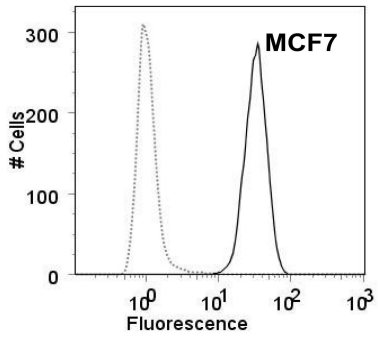
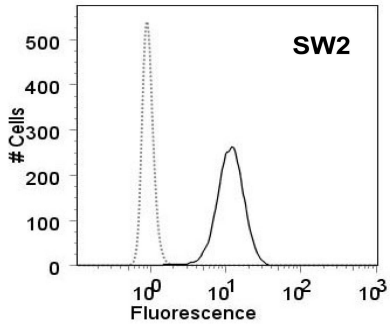
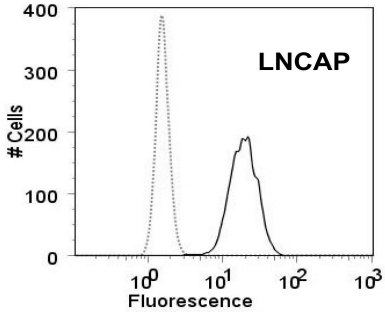
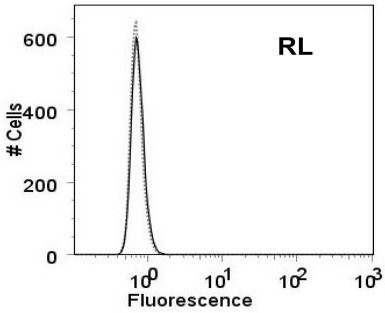
Fluorescence

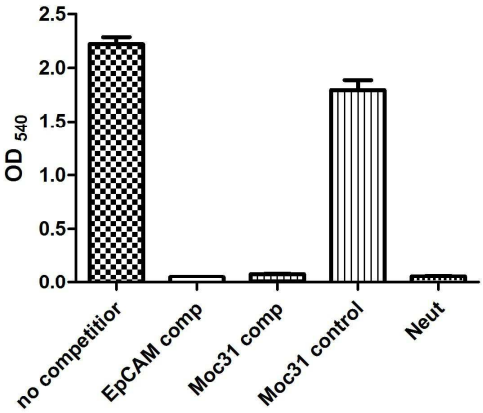

MCF7
# Cells
Fluorescence


SW2
# Cells
Fluorescence


LNCAP
# Cells
Fluorescence


RL
# Cells
Fluorescence

**D**


OD 540
no competitior
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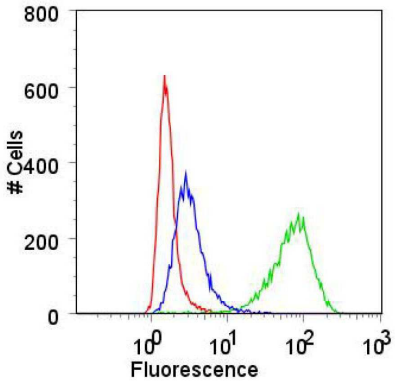

# Cells
Fluorescence

**Figure 3**. **A) ELISA of EpCAM-specific binders fused to sfGFP.** DARPins fused to superfolder GFP (sfGFP) were analyzed for EpCAM binding on ELISA. Biotinylated EpCAM (20 nM) was immobilized on neutravidin coated plates. Binding of DARPins_sfGFP was detected using a mouse anti-RGS$(His)_4$ as primary and alkaline phosphatase-coupled goat anti-mouse IgG as secondary antibody. E3_5_sfGFP, an unselected member of the library, is shown as a negative control. **B) Flow cytometry analysis of MCF-7 cells with, EPh1_sfGFP, EPh1Avi and untagged Eph1.** EPh1_sfGFP; EPh1Avi_strepAF488 (streptavidin_AlexaFluor488); and untagged EPh1 or as control an unselected member of the library E3_5 (E3_5sfGFP, E3_5Avi_strepAF88 and untagged E3_5) were incubated with MCF-7 cells for 45 minutes at 4°C. Untagged EPh1 and E3_5 were detected with anti-$(His)_4$ primary antibody and anti mouse FITC labeled secondary antibody. Cells were analyzed by flow cytometry. Red: autofluorescence of MCF-7 cells, green: E3_5_sfGFP, orange: E3_51Avi_strepAF488, blue: untagged E3_5, tinted green: EPh1_sfGFP, tinted orange: EpH1_Avi_strepAF488, tinted blue: untagged EpH1. **C) Binding of EPh1 to different tumor cell lines analyzed by flow cytometry**. EPh1 fused to sfGFP was incubated for 45 min at 4°C with different EpCAM positive carcinoma cell lines (CAL 27, HT29, MCF7, SW2, and LNCAP). The EpCAM negative cell line RL was used as control. Fluorescence signals from EPh1_sfGFP are shown in black, autofluorescence of cells in grey. **D) ELISA_ MOC31 competition.** Binding of EPh1 to immobilized EpCAM was competed with the monoclonal antibody Moc31 and with soluble non biotinylated EpCAM. Biotinylated EpCAM (20 nM) was immobilized on neutravidin coated plates. Binding of EPh1 (10 nM) was detected. Competition with a 10 fold excess of Moc31 (100 nM) or with soluble EpCAM (100nM) was examined. Moc31 (100 nM) binding to bEpEx is included as positive control. Binding of EPh1 to Neutravidin is shown as background control. **Flow cytometry_ MOC31 competition.** Binding of EPh1 to CAL27 cells was competed with the monoclonal antibody Moc31. CAL27 cells (1. $10^6$ cells) were incubated with EPh1_sfGFP alone (green) or in the presence of an excess of the monoclonal antibody Moc31 (blue). Cells autofluorescence is shown in red.

## Internalization by confocal microscopy

Since our strategy is to use DARPins for targeted delivery of cancer therapeutics to solid tumors, we explored if EPh1, the first generation molecule, is efficiently internalized upon binding to EpCAM on the cell surface. Therefore, EPh1 was labeled with Alexa Fluor 488 and added to MCF-7 tumor cells at 37°C (to allow receptor internalization) or at 4°C (to prevent internalization). Cells were observed using a confocal microscope to monitor subcellular localization of the DARPin (Figure 4). The staining pattern obtained at 4°C showed predominantly diffuse surface membrane fluorescence, whereas at 37°C the bright punctuated dots represented internalized DARPin, which was probably localized in endo-/lysosomes.

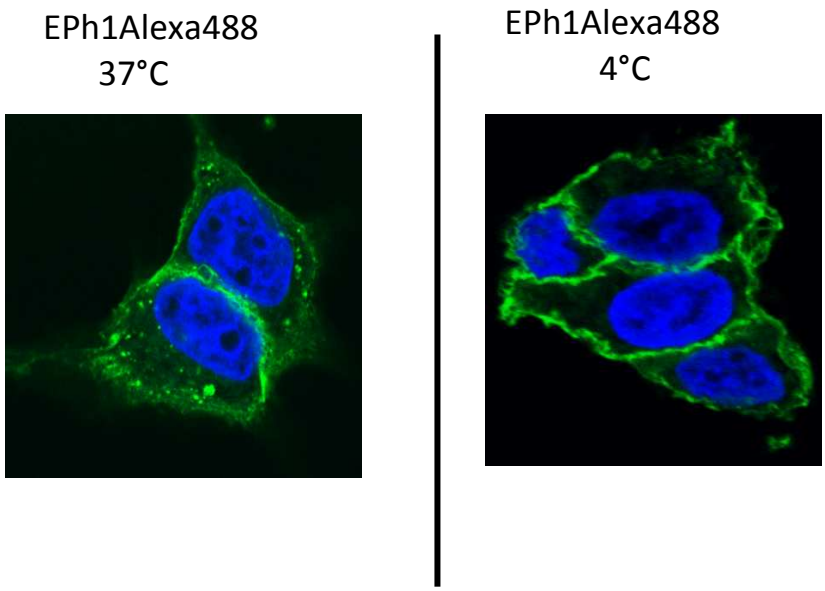



**Figure 4. EPh1 internalization by confocal microscopy.** The EpCAM-positive cell line MCF-7 was treated with fluorescent labeled EPh1 at 37°C or 4°C. After 1 hour incubation, cells were washed, fixed with 4% PFA, and then imaged using a confocal scanning fluorescence microscope. Nuclei were counterstained with DAPI. The images were produced with a selected confocal plane in the approximated middle of the cells. Images were processed using Imaris 3D software

## Selection of EpCAM-specific DARPins using ribosome display

To increase our range of binders, two different DARPin libraries (N2C and N3C) (19) were also used in ribosome display. Three selection rounds in solution were performed and DARPins obtained after the third round of selection were analyzed for specific binding to bEpEx by crude extract ELISA and we identified seven EpCAM binders which belong to three distinct families, defined by having the same or almost the same randomized positions but different framework mutations. All sequences differed at the randomized positions from EPh1. The newly selected DARPins were fused to sfGFP and analyzed for binding to EpCAM by flow cytometry. All analyzed DARPins showed binding to EpCAM-positive but not to EpCAM-negative cells (data not shown). While it is tempting to speculate that the greater size of the ribosome display library, and the inherent diversification by random mutations in the ribosome display process, contributed to the larger number of cell-binding clones, it is also possible that epitope masking employed in phage display significantly restricted the accessible space. Also, the ribosome display selection was carried out in solution, perhaps better preserving native epitopes and not exposing new ones.

The mean fluorescence intensity (MFI), measured by FACS, of the newly selected DARPins was lower when compared to the MFI of EPh1 (all tested at the same concentration) (data not shown). This difference could be due to lower functional affinities to EpCAM, either intrinsic, or cause by different accessibilities of the epitopes recognized.

## Affinity maturation

EPh1 and the previously ribosome display selected binders were then subjected to affinity maturation by using error-prone DNA polymerase in the presence of dNTP analogs (36) followed by new rounds of selection using ribosome display.

The binders initially selected from ribosome display were in the mid-nanomolar range as estimated from competition ELISA (37) and data not shown, and their affinity needed therefore to be increased. In contrast, EPh1 showed much higher affinity already, but it gave rise to formation of dimers or multimers. We obtained evidence (see below) that this was not due to any instability of the molecule, but most likely to fortuitous self-complementarity of its surface. We were therefore interested in whether this self-complementarity could also be removed by directed evolution.

For this purpose, we first replaced the C-cap of all EpCAM binders with a more stable version that was recently designed (23). After error-prone PCR, the pools were subjected to four or five rounds of selection by ribosome display using a combination of stringent and non-stringent rounds (30). On the basis of crude extract ELISA signals and SDS PAGE analysis, 92 clones were sequenced, resulting in 65 unique sequences. Sequence analysis revealed that thirty sequences (46%) were derived from EPh1: this group of binders maintained the same randomized positions and the characteristic two amino acid deletion as EPh1, but had different framework mutations. The rest of the affinity matured binders (33 sequences or 51%) were derived from different binders that had come from the original ribosome display selection. Two binders could not be assigned to any family of input binders.

All 65 affinity-matured binders were expressed and purified in 96-well format. Based on competition ELISAs, analytical size exclusion chromatography and flow cytometry analysis we chose the six best EpCAM binders. Four binders belonged to the EPh1 family and were named Ec1 to 4, the other two binders were derived from a binder selected by the original ribosome display selection and were named Ac1 and Ac2.

## Characterization of the evolved final six EpCAM binders

Binding to EpCAM was tested by competition ELISA; consistent with specific binding, the signal in ELISA was decreased for all six binders when non-biotinylated EpCAM was used as competitor (Figure 5A).

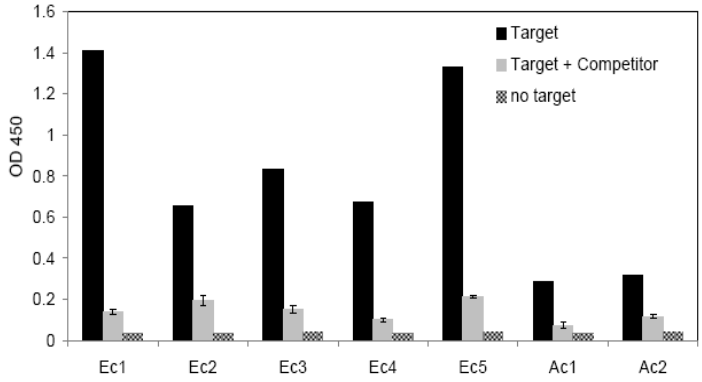
The proteins were analyzed by gel filtration. Ac1 and Ac2 were predominantly monomeric but showed a small shoulder that might correspond to a small amount of dimer (Figure 5B), but no aggregation was found. Ec1 to 4, derived from EPh1, were entirely monomeric, with no indication of any other species.

The ribosome display, intended for affinity maturation, therefore indeed increased the affinity, but also removed the dimerization and oligomerization tendency of EPh1. We therefore wished to understand the mechanism of this favorable selection outcome. EPh1 contains a two amino-acid deletion, needed for recognizing its cognate epitope, but the deletion is in the hairpin loop connecting the N-cap with the first internal repeat and can be perfectly accommodated in the structure. We analyzed the modeled structure by ROSETTA (38), but found no evidence for any unfavorable interactions by this deletion, and the expression level of this DARPin (140 mg/l) is in the range of normal DARPins. We then investigated all mutations that were selected in ribosome display, but there were none that were strongly stabilizing according to the ROSETTA score. This argues against the EPh1 deletion having destabilized the DARPin and the ribosome display selected mutations compensating for it. Instead, the data are consistent with the dimerization and multimerization of EPh1 be entirely due to a fortuitous self-complementarity of the surface of native DARPin molecules, and the evolution process having removed some interacting residues and thus leading to monomers, at the same time leading to picomolar affinity to the target EpCAM

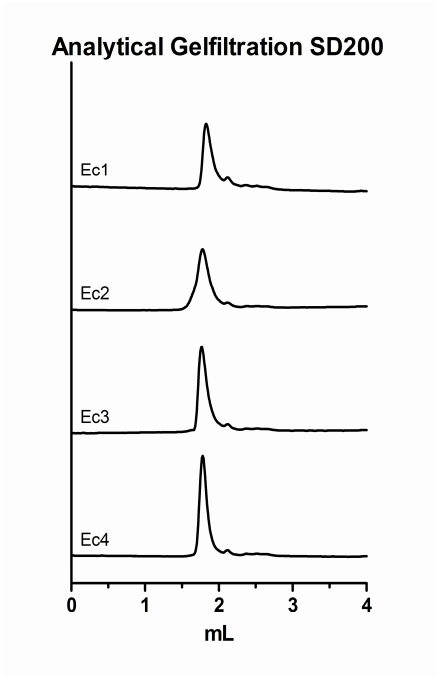
The ability of the six DARPins to interact with EpCAM on the cell surface was determined by flow cytometry. All DARPins bound to EpCAM-positive cells but not to EpCAM-negative control cells. The unselected DARPin E3_5 did not interact with any of the tested cell lines (Figure 5C).

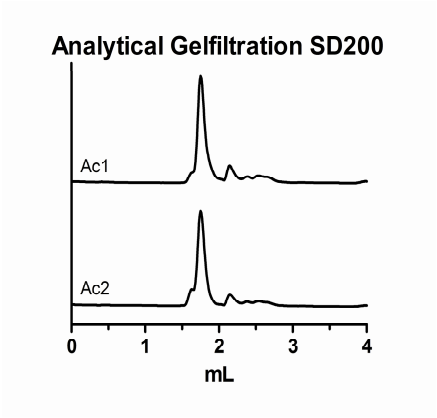
We further analyzed if the two families bind to different EpCAM epitopes by flow cytometry. To this end, binding of Ec4_sfGFP in the presence of an excess of Ac2, and binding of Ac2_sfGFP in the presence of an excess of Ec4 to MCF-7 cells was analyzed. Since no mutual competition was observed, we conclude that the two families recognize non-overlapping epitopes. Additionally, as expected Ec4, the Eph1 derived binder, competed for binding with the EpCAM antibody, MOC31, while Ac2 did not (data not shown).

**A**




**B**



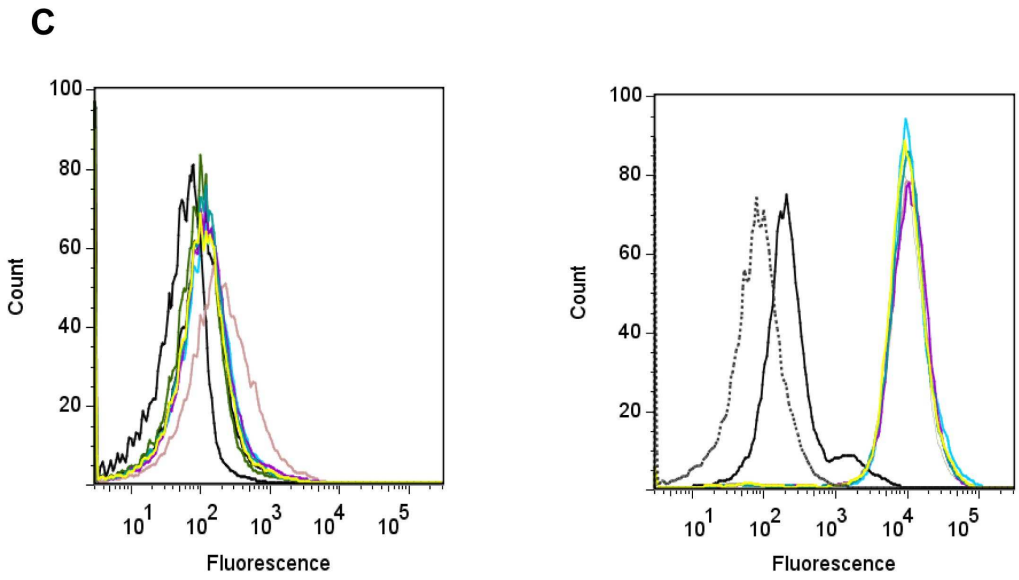



**Figure 5. A) Competition ELISA binding assay.** Biotinylated EpCAM (10 nM) was immobilized on streptavidin-coated plates. EpCAM-binders (10 nM) were allowed to bind for 10 min. For competition, binders were preincubated with a 10-fold excess of non-biotinylated EpCAM. Detection was done with mouse anti-RGS(His)$_4$ as primary and alkaline phosphatase-coupled goat anti-mouse IgG as secondary antibody. **B) Analytical gel filtration.** 15µM DARPin (50 µL) were analyzed on an Ettan™ liquid chromatography system using a Superdex™ 200 column (both GE Healthcare). **C) Analytical flow cytometry**. EpCAM-positive SW2 cells were incubated at 4°C with the EpCAM-specific DARPins for 1h. Detection with mouse anti-RGS(His)$_4$ as primary and Alexa Fluor 488 coupled goat anti-mouse IgG as secondary antibody. Blue: autofluorescence of SW2 cells, red: unselected library member (negative control), rest: EpCAM-specific DARPins. Specificity of depicted DARPins was confirmed with EpCAM-negative cells RL. Blue: autofluorescence of RL cells, rest: EpCAM-specific DARPins.

## Affinity determination of Ec4

We determined the dissociation constant ($K_D$) of Ec4, by surface plasmon resonance (SPR) with purified bEpEx as 920 ± 80 pM.

We also wished top determine the affinity for EpCAM on cells, and therefore used flow cytometry of Ec4_sfGFP on MCF-7 cells, using the determination of association and dissociation rates on cells (39) under conditions where internalization was minimized. The calculated functional affinity on cells was 7.9 ± 0.5 nM (Figure 6).

The difference in affinities to SPR measurements was entirely due to an almost 10-fold slower $k_{on}$, whereas the dissociation rate was identical in both experimental setups. The slower association is not caused by the bigger size of the DARPin-GFP fusion, as the same binder coupled to Alexa Fluor 488 bound to cells at a similar rate. Therefore, we suggest that this lower on-rate is due to a limited access of the cognate epitope on the cell surface.

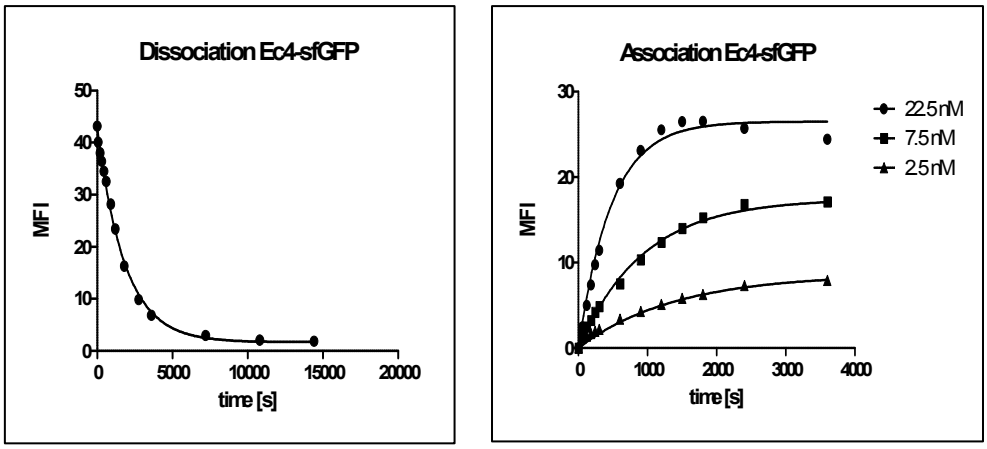



**Figure 6. Association and dissociation of Ec4-sfGFP from EpCAM-positive MCF-7 cells.** For dissociation, cells were incubated with 100 nM Ec4_sfGFP for 1h at 4°C, washed, resuspended in 1 µM Ec4 (t=0) to prevent rebinding. For association, cells were resuspended in 2.5, 7.5 and 22.5 nM Ec4-sfGFP (t=0). Mean Fluorescence intensities measured on a FACScanto II analytical flow cytometer (BD Biosciences) at different time points were used to calculate $k_a$, $k_d$ and$K_D$.

## Discussion

The aim of this study was the generation of specific binders from a DARPin library against the tumor-associated antigen EpCAM. EpCAM is widely expressed on solid tumors and upon ligand binding, it is efficiently internalized by receptor-mediated endocytosis. Thus, it perfectly matches the need for intracellular delivery of anticancer agents acting on intracellular targets such as DNA-damaging agents, toxins, antisense oligonucleotides and siRNA (14, 16-17, 37).

The extracellular domain of EpCAM was expressed in HEK293T cells and used as target protein for our DARPin selections, as it is glycosylated in several positions (40). We employed two different selection techniques, SRP phage display and ribosome display with the purified protein. Both selection techniques were found to be well-suited for the *in vitro* selection of binders from synthetic DARPin libraries.

After the initial selection by phage display on immobilized target we found one dominant binder, named EPh1, having a characteristic two-amino-acid deletion between the N-terminal capping repeat and the first internal repeat. This deletion was not observed in other selections performed with the same library. It appears to favor binding to a particular epitope, since when the two amino acids were reinserted, binding affinity was significantly reduced. Selection of only one dominant binder or a low diversity of binders selected on certain target proteins is a common phenomenon often observed with phage display (29). In this case, the deletion — which most likely happened in the original assembly of the library from synthetic oligonucleotides — is a very rare event, leading in this case to a very valuable clone, for which there was no alternative. This particular clone appeared to show fortuitous self-complementarity, which could be completely removed during a further maturation by ribosome display, showing the great powers of evolutionary optimization. We assume that no equivalent binder was found be selecting directly from the ribosome library since no similar 2-amino-acid deletion molecule may have been present in the ribosome display libraries.

To overcome this limitation, we employed epitope masking to recover binders recognizing other epitopes (32). The five new binders so obtained specifically recognized EpCAM in ELISA failed to bind to EpCAM expressed on the cellular surface, and only EPh1 was capable of binding to EpCAM expressed on different tumor cells. It is possible that during the selection procedure, soluble EpCAM may have exposed additional epitopes which are normally hidden when EpCAM is anchored in the cell membrane. Additionally, blocking the preferred epitope may make even closely adjacent ones inaccessible, thereby directing binding to epitopes too close to the cell membrane, or those that are inaccessible by oligomerization of EpCAM (35), or EpCAM interaction with other molecules on the cell surface. EpCAM was shown to interact with the tight junction protein claudin-7 followed by recruitment into tetraspanin-enriched membrane microdomains where it is complexes with tetraspanin and CD44v6 (9).

Ribosome display selection using an N2C and an N3C DARPin library (19) resulted in seven new binders that belong to three families. None of these families featured the same randomized positions as EPh1, nor its 2-amino-acid deletion. All binders selected by ribosome display were capable of binding to EpCAM in its native conformation on cells. One of these DARPins, C9, was tested to explore its potential use for tumor-targeting (37). However, its affinity was in the mid-nanomolar range and therefore, it was also converted to different dimeric forms, and fused to protamine, a highly positively charged peptide that can complex small interfering RNA. These fusion proteins efficiently delivered siRNA complementary to *bcl-2*, a potent inhibitor of apoptosis implicated in cancer drug resistance and successfully down-regulated *bcl-2* expression in an EpCAM-specific manner, facilitating cell-specific apoptosis (37).

We then used affinity maturation to improve all previously selected binders by combining error-prone PCR with stringent selection by ribosome display (26, 30). Of the finally chosen 6 binders, two were derived from the ribosome display pool and four of the binders were derived from Eph1 selected by SRP phage display. Members of this group maintained not only the same randomized positions but also the characteristic two-amino-acid deletion found in EPh1 and had four to six different framework mutations. These framework mutations eliminated any self-association tendency that the precursor EPh1 had, and these proteins were pure monomers. An analysis by ROSETTA gave no

indication that EPh1 had any problematic amino acids that were "corrected", and it thus directed evolution appears to have eliminated surface features that have allowed self-complementarity.

One of the evolved binders Ec4 was characterized in more detail. Its binding on cells showed a slower on-rate than measured by SRP, leading to low nanomolar $K_D$ on cells, while it is subnanomolar in SPR measurements. Ec4 similarly to their parental binder EPh1, is internalized into cells upon binding to EpCAM, which is a mandatory prerequisite for the delivery of therapeutic molecules acting on intracellular targets.

Since we determinated that Ec4 recognize a different epitope on EpCAM than Ac2 (another evolved binder), we were able to further enhance the functional affinity of the binders on cells by generating dimers and tetramers of the two DARPins by fusion to a self-associating peptide. Preliminary data show that this multimerization enhances the binding activity due to an up to 100-fold decrease in the dissociation rate (N Stefan, unpublished)

In summary, we found merit in using both phage display and ribosome display to select binders for a demanding highly glycosylated target which may present only few accessible epitopes. By combining both selection methods and subsequent stringent directed evolution we generated high affinity binders to non-overlapping epitopes of EpCAM. These DARPins expressed at high yield and could be easily purified. Since they can be linked in many new formats and are efficiently internalized, they may provide the basis for a new generation of protein therapeutics, which are superior to antibodies in terms of stability, production, biophysical properties and freedom for rational engineering and optimization.

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**Chapter 3**

# A novel Fusion Toxin Derived from an EpCAM-specific Designed Ankyrin Repeat Protein has a Potent Anti-tumor Activity

**Submitted article**

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# A Novel Fusion Toxin Derived from an EpCAM-specific Designed Ankyrin Repeat Protein has Potent Anti-tumor Activity

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## Abstract

*Purpose*: Designed Ankyrin Repeat Proteins (DARPins) hold great promise as a new class of binding molecules to overcome the limitations of antibodies for biomedical applications. Here, we assessed the potential of an EpCAM-specific DARPin (Ec4) for tumor targeting in form of a fusion toxin with *Pseudomonas* exotoxin A.

*Experimental design:* DARPin Ec4 was genetically fused to a truncated form of exotoxin A (ETA") and expressed in *E.coli*. The cytotoxicity of Ec4-ETA" was measured against tumor cell lines of various histotypes *in vitro.* Tumor localization and anti-tumor activity were determined in athymic mice bearing EpCAM-positive tumor xenografts.

*Results:* Ec4-ETA" expressed very well in soluble form in the cytoplasm of *E. coli* and yielded up to 40 mg after purification per liter culture. The protein was shown to be monomeric and the disulfides of ETA" formed spontaneously. Ec4-ETA" bound to EpCAM with picomolar affinity, similar to free Ec4. Furthermore, it was highly cytotoxic against various EpCAM-positive tumor cell lines *in vitro* with $IC_{50}$ values less than 0.005 pM. This effect was competed by free Ec4, but not unspecific DARPins. Upon systemic administration in athymic mice, Ec4-ETA" efficiently localized to EpCAM-positive tumors to achieve maximum accumulation 48-72 h after injection, whereas an irrelevant control fusion toxin did not. Tumor targeting with Ec4-ETA" resulted in a strong anti-tumor response including complete regressions in some animals.

*Conclusions:* Our data demonstrate for the first time the potential of DARPins for the generation of protein therapeutics for tumor targeting, and that Ec4-ETA" deserves attention for clinical development.

## Introduction

The concept of tumor-targeted therapy is based on the use of conjugates consisting of ligands binding to tumor-associated antigens or growth factor receptors which deliver cytotoxic agents selectively to tumors, while sparing normal tissues from destruction (1). These agents include radioisotopes, small organic compounds, antisense oligonucleotides and protein toxins. All of them exert different modes of action, compared to standard chemotherapy, and thus might be particularly useful to combat drug-resistant cancer. Nonetheless, it remains to be shown in every single case whether a tumor-specific localization actually occurs and what profile of anti-tumor action compared to side effects is seen.

Immunotoxins are a class of conjugates in which antibodies or antibody fragments are chemically linked to protein toxins, whereas in the more advanced constructs targeting ligand and toxin are genetically fused (2). The most popular toxins used for this purpose are diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A (ETA) (3), which both act by irreversibly inhibiting protein synthesis in cells. In ETA-based fusion toxins a truncated variant lacking the cell binding domain and carrying a C-terminal KDEL peptide (denoted here ETA") is commonly used (4). Currently, several of these fusion toxins are in clinical trials for the treatment of lymphomas, leukemias (5-6), mesothelioma and cancers of the ovary, pancreas and bladder (7-8).

On solid tumors, well investigated targets for antibody-based therapies are members of the epidermal growth factor receptor family such as EGFR itself and ErbB2, and certain tumor-associated carbohydrates (2, 9). Recently, the epithelial cell adhesion molecule (EpCAM) has also emerged as a promising structure for targeted therapy of solid tumors. One reason is that its efficient internalization promotes access of surface bound effector molecules to intracellular targets (10-13). EpCAM is a homophilic cell adhesion molecule of 39 to 42 kDa, consisting of an extracellular domain with an epidermal growth factor-like and a human thyroglobulin-like domain, and a short cytoplasmic domain. Its processing by regulated intramembrane proteolysis releases a cytoplasmic tail

which activates the *wnt* signaling pathway and induces transcription of c-myc and cyclins (14-15). How this mechanism contributes to tumor progression *in vivo* is unclear. EpCAM is expressed at low levels on basolateral cell surfaces of some normal epithelia where it is, however, poorly accessible to circulating anti-EpCAM antibodies (16). On the other hand, high levels of homogenously distributed EpCAM are detectable on cells of epithelial tumors (14, 17), and its overexpression represents an independent prognostic marker for reduced survival in patients with breast and ovarian cancer (18-19). Recently, EpCAM was also identified as a marker of cancer-initiating cells in colon (20), breast (21) and pancreatic cancers (22), providing the opportunity to target stem-like cells, which usually respond poorly to standard therapy. The favorable properties of EpCAM for cancer therapy are currently exploited in phase II clinical trials with a scFv-ETA" immunotoxin (8, 10-13) which we developed previously.

Nowadays, the tumor-targeting moiety for the delivery of cytotoxic agents including protein toxins is usually derived from antibodies or antibody fragments, which, however, have practical limitations due to their poor expression yield and aggregation tendencies, at least for some constructs (23-24). Since for fusion toxins no other feature of the antibody is required than antigen binding, a solution might come with the use of alternative non-IgG binding scaffolds as targeting moieties. These can be engineered for improved specificity, affinity, and stability to increase the production yield. One such protein class are Designed Ankyrin Repeat Proteins (DARPins) (25-27). The ankyrin repeat motif consists of 33 amino acids forming a loop, a β-turn and two antiparallel α-helices connected by a tight turn. Their high stability and favorable biophysical properties provide proteins which tolerate engineering procedures usually not applicable to antibodies. Moreover, they contain no cysteine which can instead be introduced for site-specific modifications. Thus, DARPins fulfill the requirement of almost ideal candidates for many biomedical applications including tumor targeting. Using combinatorial libraries of DARPins along with selection by ribosome or phage display, we recently generated several binders specific for EpCAM, and demonstrated their potential for efficient delivery of therapeutic siRNA into tumor cells (Martin Killias et al., in preparation,(13).

Here we describe for the first time the use of a high affinity DARPin (Ec4) specific for EpCAM to generate a fusion toxin with ETA". Ec4-ETA" expressed very well in *E. coli,* was easily purified to high yields, and proved to be specifically

cytotoxic against various EpCAM-positive tumor cell types *in vitro*. *In vivo*, fluorescence imaging and therapy studies in athymic mice demonstrated its ability to efficiently localize to subcutaneously growing tumors upon intravenous administration, and induce strong anti-tumor effects including complete regressions.

## Translational Relevance

Antibodies or antibody fragments are widely used as targeting moiety for the delivery of cytotoxic drugs to tumors. However, many of these constructs have limitations due to their poor expression and aggregation tendency. To overcome these limitations, we used for the first time Designed Ankyrin Repeat Proteins (DARPins) as non-IgG scaffolds with favorable biophysical properties and much higher expression yield in *E. coli* and systematically tested its potential for tumor-targeted delivery of a highly potent biotoxin in preclinical studies. The fusion toxin Ec4-ETA" recognizing the carcinoma-associated antigen EpCAM was potently cytotoxic against carcinoma cell lines of various histotypes *in vitro.* In athymic mice targeting of human carcinoma xenografts with Ec4-ETA" resulted in a strong anti-tumor response including complete regressions. This demonstrates that DARPins are well suited for tumor targeting and that the fusion toxin Ec4-ETA" holds promise for clinical development.

## Material and Methods

### Tumor cells

The squamous cell carcinoma cell line of the tongue CAL27 and the colorectal carcinoma cell line HT29 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen, Braunschweig, Germany). The breast carcinoma cell line MCF7 and the non-Hodgkin's lymphoma cell line RL were obtained from ATCC (American Type Culture Collection). The small cell lung carcinoma cell line SW2 was maintained in our laboratory. All cells were cultured in Dulbecco's modified medium (DMEM) (Sigma, Buchs, Switzerland), supplemented with 10% fetal calf serum (Amimed, Bioconcept, Allschwil, Switzerland), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma). Cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. All cells were tested negative for mycoplasma using MycoAlert (Lonza, Basel, Switzerland).

### Construction, expression and purification of DARPin-ETA" fusion toxins

The EpCAM-specific high affinity DARPin Ec4 was selected from a DARPin library as described (Martin Killias et al., in preparation), the control DARPins off7 (targeting the maltose binding protein) and E3_5 (an unselected member of the N3C library) have been described elsewhere (26-27). The sequences encoding the DARPins were inserted via *Bam*HI and *Hin*dIII upstream into a expression vector derived from pQE30 , containing a 12 amino acid linker $(GSG_4)_2$ and the 40 kDa truncated form of $ETA_{252\text{-}608}$KDEL (ETA") which was cloned as described (10, 28). ETA comprises residues Glu252-Pro608 (numbering of the mature protein), fused to a C-terminal his tag followed by KDEL (denoted $ETA_{252\text{-}608}$KDEL or ETA").

For purification and detection the construct in addition contains an MRGS-$His_6$ tag at the N terminus. The DARPin-ETA" fusion proteins were expressed in soluble form in *E. coli* BL21(DE3) strain (Stratagene, La Jolla, USA). Cultures were harvested 4 h after induction with 1 mM IPTG. For purification the bacteria were resuspended in $TBS_{400}$ (50 mM Tris, 400 mM NaCl, pH 7.4 at 4°C) with 20 mM imidazole and lysed with a TS 1.1 kW cell disruptor (Constant Systems Ltd., Northants United Kingdom). Upon centrifugation (48000 *g*, 30 min

at 4°C) and filtration (pore size 0.22 µm), the fusion toxins present in the clear supernatant were purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA superflow (Qiagen, Hilden, Germany).

**Endotoxin removal**

For *in vivo* application, the DARPin-ETA" fusion toxins were further purified to eliminate endotoxin. To this end, an additional washing step with 150 column volumes PBS containing 20 mM imidazole and 0.1% Triton-X-114 was performed during Ni-NTA purification, followed by size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare, Zürich, Switzerland). Monomeric fractions were further depleted of residual endotoxin by passage over an EndoTrap Red column (Hyglos, Regensburg, Germany), and the final endotoxin content was determined using a commercially available Limulus amebocyte lysate (LAL) endochrome kit (Charles River, Sulzfeld, Germany).

**Measurement of EpCAM-binding affinity**

The EpCAM-binding affinity of Ec4-ETA" was measured by surface plasmon resonance using a Biacore 3000 (GE Healthcare) instrument. A streptavidin (SA) chip was coated with 120 resonance units (RU) of biotinylated extracellular domain of EpCAM (residues 1 to 242 of the mature protein). Measurements were performed by serial injection of different concentrations of Ec4-ETA" ranging from 10 to 160 nM at a buffer flow of 30 µl/min in HBST (20 mM Hepes, pH 7.4, 150 mM NaCl, 3mM EDTA, 0.005% Tween-20). Data evaluation was performed using the BIAEVAL software (GE Healthcare) and Scrubber 2 (BioLogic Software, Campbell, Australia).

**Disulfide assays**

The formation of the two disulfide bonds in Ec4-ETA" after purification of protein expressed in the cytoplasm was quantified according to (29). Briefly, 1.25

nmol of fusion protein was treated with 4,4'-dithiodipyridine (4-DPS) and compared to a sample reduced with sodium borohydride, quantified by HPLC and evaluated according to a standard curve made with known amounts of cysteine.

**Cytotoxicity assays**

The specific cytotoxic activities of the DARPin-ETA" fusion toxins were assessed by measuring cell viability in standard colorimetric XTT assays (XTT = (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tretrazolium-5-carboxanilide) sodium salt) (Roche, Basel, Switzerland). Briefly, EpCAM-positive or negative tumor cells were seeded at 5000 cells per well in a 96-well plate and incubated overnight at 37°C under standard cell culture conditions as described above. The fusion proteins were added to the cells at the indicated concentrations to a final volume of 100 µl. After 72 h, 50 µl of XTT reagent was added as specified by the manufacturer's protocol and cells were further incubated for 2 h. The absorbance at 450 nm was measured with a HTS 7000 plus microplate reader (Perkin Elmer, Wellesley, USA) and cell viability was calculated after subtraction of blanks (wells without cells) as the percentage of living cells in treated wells relative to untreated cells (cells without DARPin-ETA" toxin).

For competition analysis of specificity, cells were first preincubated for 10 min with unconjugated DARPins at the concentrations indicated before Ec4-ETA" was added and viability was determined as described above.

**Fluorescence labeling of DARPin-ETA" fusion proteins with Cy5.5**

Ec4-ETA" and off7-ETA" were incubated with a 3-fold molar excess of Cy5.5 NHS ester (GE Healthcare) pH 7.4 for 1.5 h at room temperature. The samples were passed over a PD-10 column (GE Healthcare) to remove unreacted dye and exchange the buffer to 100 nM sodium-bicarbonate with 20 nM NaCl at pH 8. At this relatively low pH the N-terminal amino group is favored over lysine residues. Monolabeled fusion proteins were separated from unlabeled and multiple labeled proteins by anion exchange chromatography on a MonoQ

column (GE Healthcare) in 100 nM sodium-bicarbonate and 1 M NaCl at pH 8 for isocratic elution.

**Animals and tumor xenografts**

For *in vivo* experiments, 8-10 weeks old female athymic mice (NMRI nu/nu, Harlan Laboratories, B.V., The Netherlands or CD1, Charles River, Sulzfeld, Germany) were used. Mice were housed and maintained under specific pathogen free conditions according to the guidelines of the veterinary offices of the Kanton Zürich and Bern. Tumors were raised by subcutaneous (s.c.) injection into the lateral flank of HT29 cells ($10^7$ cells in 100 µl PBS).

***In vivo* fluorescence imaging**

Ten days after s.c. tumor cell injection, mice were intravenously (i.v) injected with 30 µg of Ec4-ETA" or off7-ETA" conjugated with the fluorescent dye Cy5.5 (n=3 for each group). *In vivo* imaging was performed 6, 24, 48, 72, and 96 h after injection. During imaging mice were anaesthetized by intraperitoneal (i.p.) injection of body-weight adapted doses of 10% ketamine and 2% xylazin. In addition, 48 h after i.v. injection one mouse of each group was euthanized and fluorescent images of dissected organs were obtained. Images were acquired using the NightOwl II NC100, Type LB 893, 2006“ imaging system (Berthold Technologies, Bad Wildbad, Germany) with an exposure time of 60 s. For colocalization of the fluorescent image on the animal body, gray scale and pseudocolor images were merged. Quantification of signal intensity in all animals was performed with WinLight32 Software.

**Anti-tumor activity *in vivo***

Mice bearing established tumors of 50 to 100 $mm^3$ in size were i.v. injected on days 1, 3 and 5 with either 30 µg or 20 µg Ec4-ETA" or with 30 µg off7-ETA" in 100 µl PBS. Mice treated with PBS at the same time points were used as control. Treatment with the Ec4-ETA" lower dose (20 µg) was repeated

again on days 9, 13 and 15. Animals were monitored for tumor growth by caliper measurement of the shortest diameter and the longest perpendicular diameter. Tumor volume was calculated according to the formula (short diameter)$^2$ x (long diameter) x 0.4. Mice were euthanized when tumors reached a volume of 1500 mm$^3$ or when tumors showed skin ulcerations.

### *In vivo* toxicity determination

During treatment with the fusion toxins animals were controlled daily for weight loss or other signs of toxicity and discomfort (apathy, ungroomed appearance, dehydration, etc). Liver toxicity was assessed post mortem by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in plasma samples. Blood samples from 2 mice treated with PBS and 3 mice treated with 30 µg Ec4-ETA" three times every second day were collected 24 h after the final injection. Upon plasma separation, ALT and AST activities were measured photometrically.

### Statistical analysis

All data represent the mean ± SD. Statistical analyses of *in vivo* tumor growth were performed using the Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

## Results

### Expression and purification of DARPin-ETA" fusion proteins

The EpCAM-specific high-affinity DARPin Ec4 (Martin Killias et al., in preparation) and the control DARPins off7 (26) and E3_5 (27) were fused via a $(GSG_4)_2$ linker to a truncated form of *Pseudomonas* exotoxin A (residues Glu252-Pro608, numbering of the mature protein), containing a C-terminal KDEL (denoted $ETA_{252-608}$KDEL or ETA") to increase cytotoxicity in mammalian cells (30). For purification and detection, the constructs further contained a $His_6$ tag at the C-terminus in front of the KDEL sequence and a RGS$His_6$ tag at the N-terminus.

All DARPin-ETA" fusion proteins were expressed in soluble form in *E. coli* at 37°C. The protein yield was up to 40 mg/liter of bacterial culture. Purification was achieved using immobilized metal ion affinity chromatography (IMAC), which for in vivo experiments was followed by extensive Triton X-114 washing, size exclusion chromatography and an EndoTrap column to remove endotoxin. The fusion toxins showed a band at the predicted molecular weight of around 59 kDa when analyzed on SDS-PAGE (Figure 1A), and size exclusion chromatography revealed a mainly monomeric fraction (Figure 1B). Figure 1C shows a computer model of Ec4-ETA". ETA" contains two disulfide bonds, and although the fusion toxins were expressed in the bacterial cytoplasm, quantitative disulfide assays (see Materials and Methods) revealed that > 90% of the protein had both disulfide bonds formed after purification, most probably by spontaneous air oxidation (data not shown). We conclude from these findings that DARPins are well suited for the easy and cost-effective production of fusion toxins for tumor targeting.

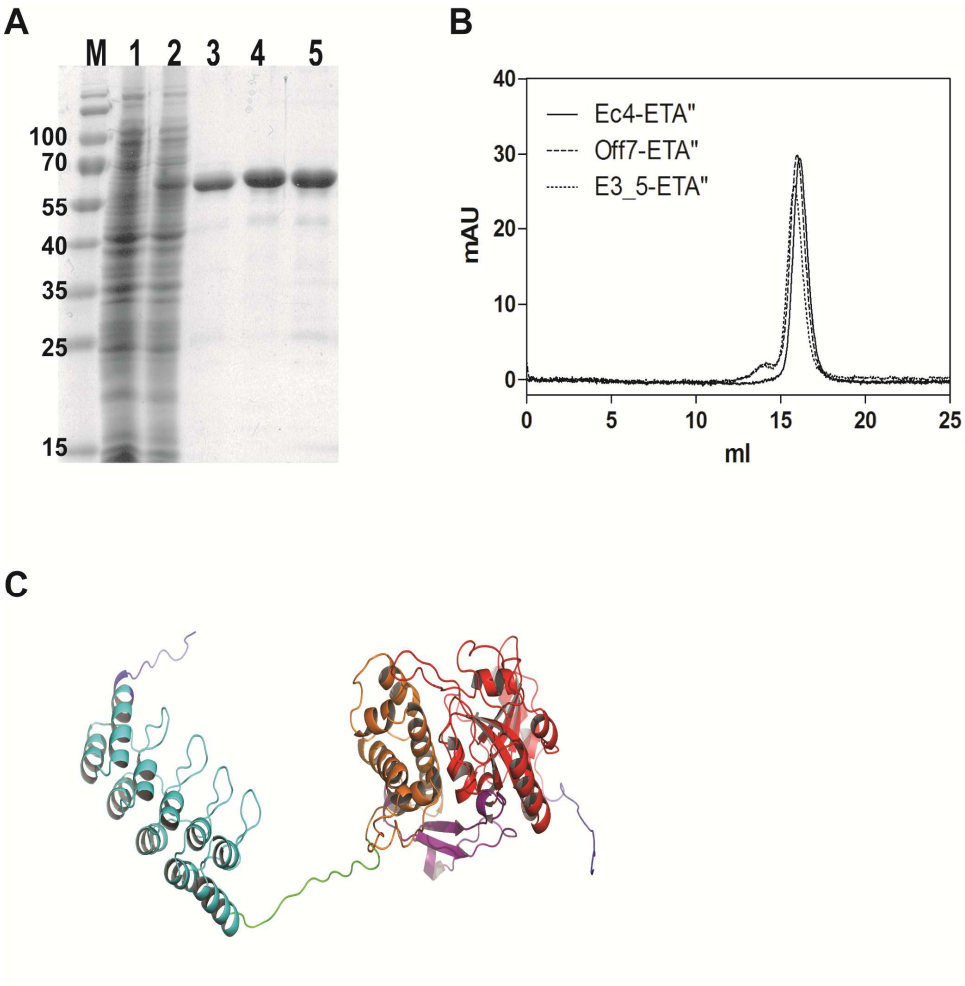



**Figure 1. Biochemical characterization of the DARPin-ETA" fusion protein toxins.**. Biochemical characterization of the DARPin-ETA" fusion toxins. **A** For SDS-PAGE analysis, fractions were loaded onto a 10% polyacrylamide gel and proteins were detected by staining with Coomassie brilliant blue: lane M: molecular weight marker (kDa), lane 1: uninduced bacterial cell lysate, lane 2: cell lysate 4 h after induction, lane 3: purified Ec4-ETA", lane 4: purified off7-ETA", and lane 5: purified E3_5-ETA". **B** Size exclusion chromatography of DARPin-ETA" fusion toxins analyzed on a Superdex 200 10/300 GL column. **C** Model of the DARPin-toxin construct was built based on the X-ray structures of a consensus DARPin (PDB entry 2QYJ) and *Pseudomonas aeruginosa* exotoxin A (ETA) (1IKQ) using InsightII (Accelrys) and the ROSETTA suite programs. The DARPin targeting moiety is shown in cyan, ETA domain Ib in purple, domain II in orange and domain III in red. The flexible N-terminal RGS-$His_6$-tag, the C-terminal $His_6$ tag and KDEL ER-retention signal (blue) as well as the linker connecting the DARPin to the toxin (green) were modeled in to visualize their sizes relative to those of the protein domains. The figure was generated using the program PyMol (DeLano Scientific LLC, Palo Alto, CA, USA).

## EpCAM-binding affinity of Ec4-ETA"

To investigate whether the fusion to ETA" impaired the binding activity of DARPin Ec4 to EpCAM, the dissociation constant ($K_D$) of the fusion protein Ec4-ETA" was measured by surface plasmon resonance (SPR) ( Figure 2). The $K_D$ was calculated as 920 ± 60 pM, which was essentially the same as measured for the Ec4 DARPin (Martin Killias et al., in preparation).

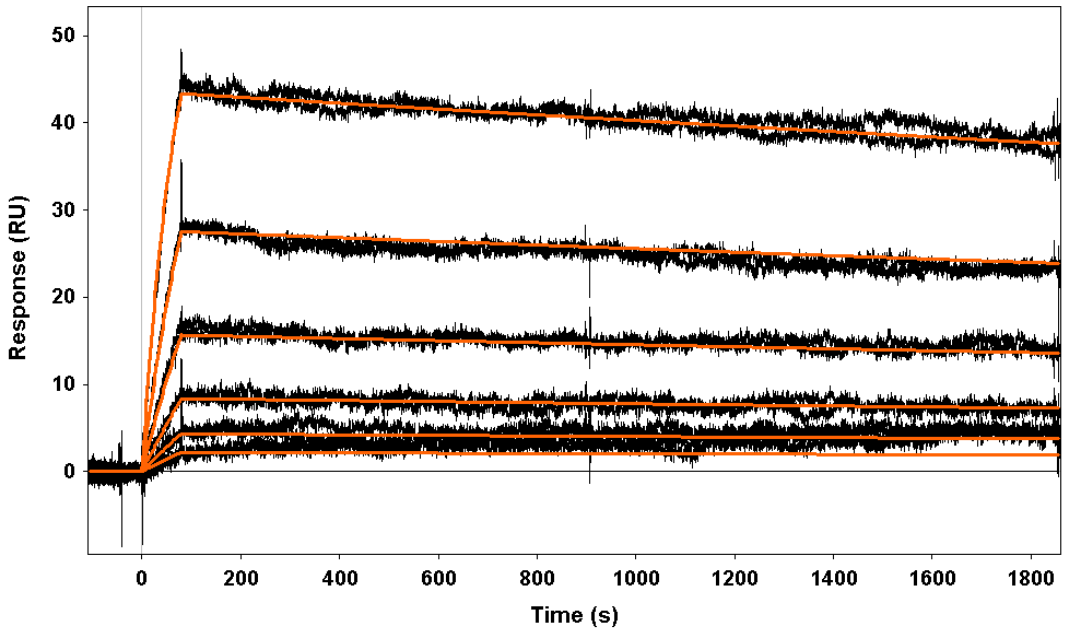



**Figure 2.** Determination of the EpCAM-binding affinity of Ec4-ETA" by SPR measurement. Enzymatically biotinylated EpCAM was immobilized on a streptavidin chip and increasing concentrations of Ec4-ETA" (5 nM, 10 nM, 20 nM, 40 nM, 80 nM, 160 nM) were assayed.Association and dissociation phases were recorded. From a measured association rate constant of 8.7 +/- 0.5 $10^4$ $M^{-1}s^{-1}$ and a dissociation rate constant of 8.0 +/- 0.08 $10^{-5}$ $s^{-1}$ a $K_D$ of 920 +/- 60 pM was calculated.

## Cytotoxicity of Ec4-ETA" against various tumor cell lines *in vitro*

The cytotoxic effect of Ec4-ETA” and the control fusion proteins off7-ETA” and E3_5-ETA” on various EpCAM-positive tumor cell lines and EpCAM-negative control cells was determined in colorimetric XTT cell viability assays upon a 72 h incubation. As shown in Fig. 3A, Ec4-ETA” was potently cytotoxic against all EpCAM-positive cell lines tested, MCF7, SW2, CAL27 and HT29. The $IC_{50}$ values (concentration at which cell viability was reduced by 50%) ranged from less than 0.005 pM to 0.7 pM. In contrast, the effect on EpCAM-negative RL cells was more than 100'000-fold lower ($IC_{50}$ > 10 nM). Similarly, the unspecific fusion proteins off7-ETA" and E3_5-ETA" showed cytotoxic effects only at much higher concentrations ($IC_{50}$ > 1 nM) when tested on EpCAM-positive HT29 cells (Figure 3B).

Furthermore, the cytotoxicity of Ec4-ETA" was markedly decreased when cells were preincubated with an excess of unfused DARPin Ec4 (Figure 3C). This decrease was specific for EpCAM blocking as preincubation with the non-specific DARPins off7 and E3_5 did not diminish Ec4-ETA" cytotoxicity. Moreover, the use of the DARPins alone did not affect cell viability (data not shown). Taken together, these data show that the cytotoxicity is mediated by EpCAM-specific uptake and background cytotoxicity by unspecific uptake of the DARPin-ETA" fusion proteins was in general very low.

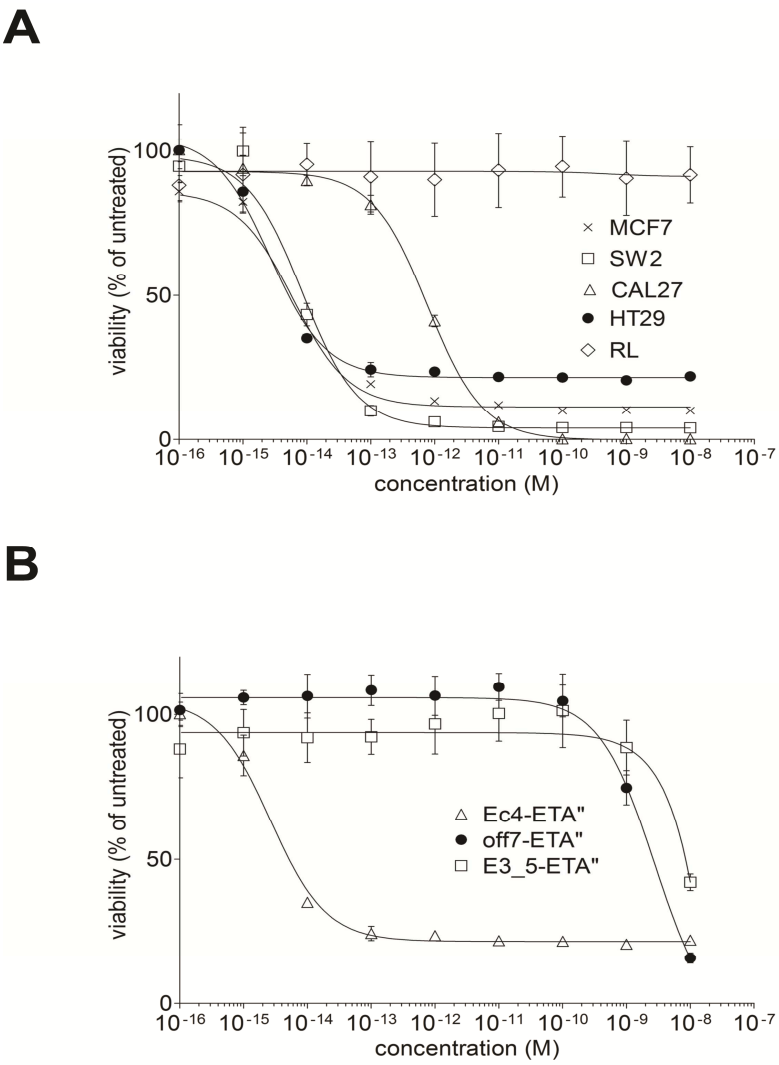



**Figure 3.** *In vitro* cytotoxicity of Ec4-ETA" and the control fusion toxins off7-ETA" and E3_5-ETA" tested on various tumor cell lines. **A** The EpCAM-positive cell lines MCF7, SW2, CAL27, HT29, and the EpCAM-negative cell line RL were incubated for 72 h with different concentrations of Ec4-ETA" before cell viability was determined in colorimetric XTT assays. **B**. HT29 cells were incubated with the unspecific fusion proteins E3_5-ETA" or off7-ETA" for 72 h in XTT assays, and viability was compared to cells treated with Ec4-ETA"

**C**

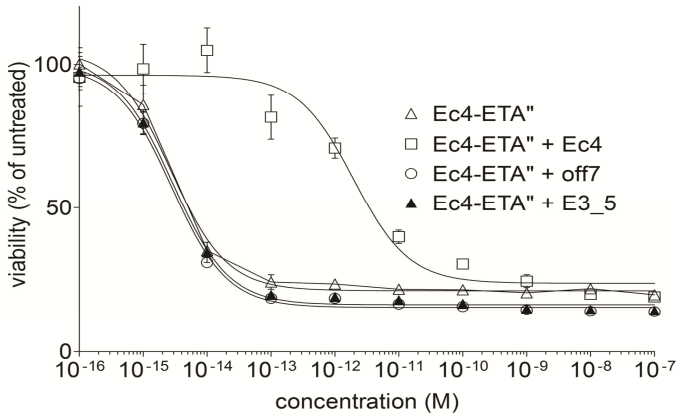
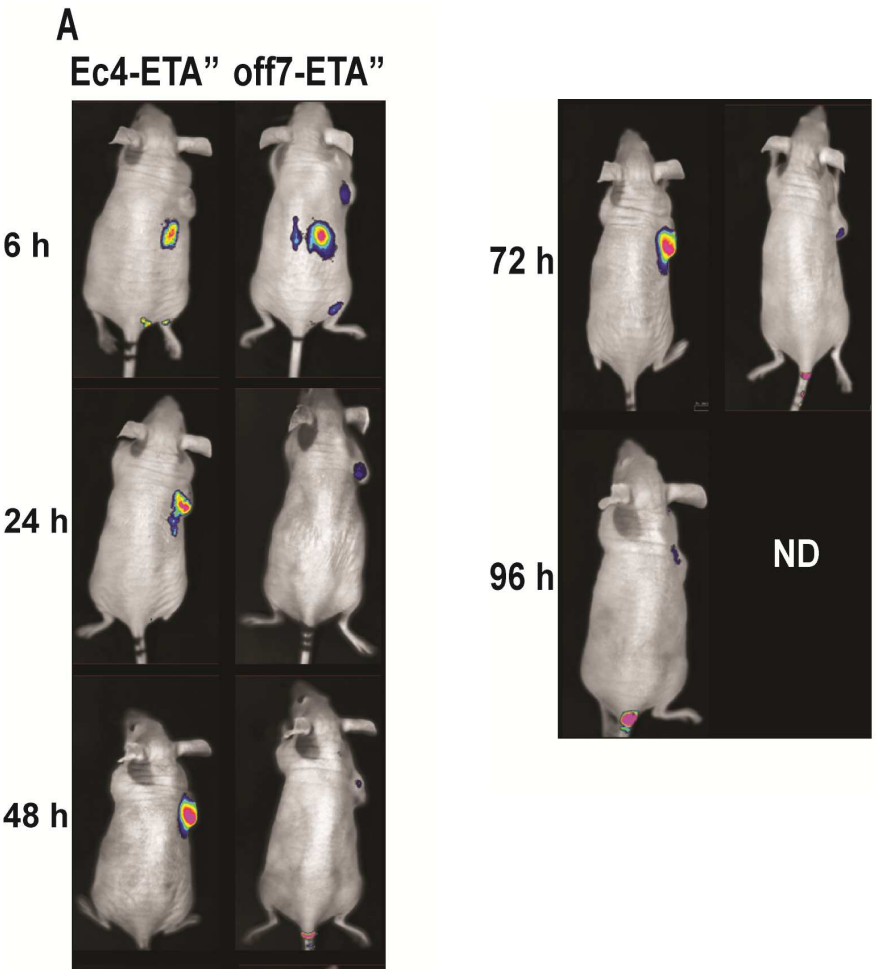



**Figure 3**. **C** In competition assays, EpCAM-positive HT29 cells were incubated with DARPin Ec4, E3_5 and off7 (500 nM) 10 min before Ec4-ETA" was added and cell viability determined as described before. All determinations were done in triplicates, data represent the mean ± SD

## Tumor localization of Ec4-ETA"

To demonstrate that Ec4-ETA" efficiently localizes to tumors upon systemic administration and that this effect is EpCAM-dependent, *in vivo* fluorescence imaging was performed in athymic mice bearing s.c. HT29 tumor xenografts. Ec4-ETA" and off7-ETA" control were N-terminally labeled with the fluorescent dye Cy5.5 (emission maximum 680 nm). After the coupling reaction, the labeled proteins were purified by anion exchange chromatography to eliminate unlabeled protein, free dye and other labeled protein species. Mice were injected i.v. with 30 µg Ec4-ETA"_Cy5.5 or off7-ETA"_Cy5.5, and images were taken after 6, 24, 48, 72 and 96 h using the NightOWL II LB891 imaging system. As shown in Fig. 4A, 6 h after injection both fusion proteins localized to the lower abdomen which could be identified as mainly kidney and partially liver accumulation. Ec4-ETA" efficiently localized to tumors 24 h after injection, and accumulation peaked between 48 h and 72 h before it declined to background values after 96 h. In contrast, only very low background fluorescence was detectable in the tumors upon injection of the non-targeted fusion protein off7-

ETA". Furthermore, we performed *ex vivo* analysis of biodistribution in isolated organs and tumors 48 h after injection of the fluorescent-labeled probe using a fiber optic device. As shown in Fig. 4B, similar to whole animal imaging high fluorescence activity in the tumor was detected only in mice injected with Ec4-ETA" but not off-7-ETA". Both fusion proteins, however, showed localization in the liver and at low levels in the intestine, which likely reflects their metabolic degradation.



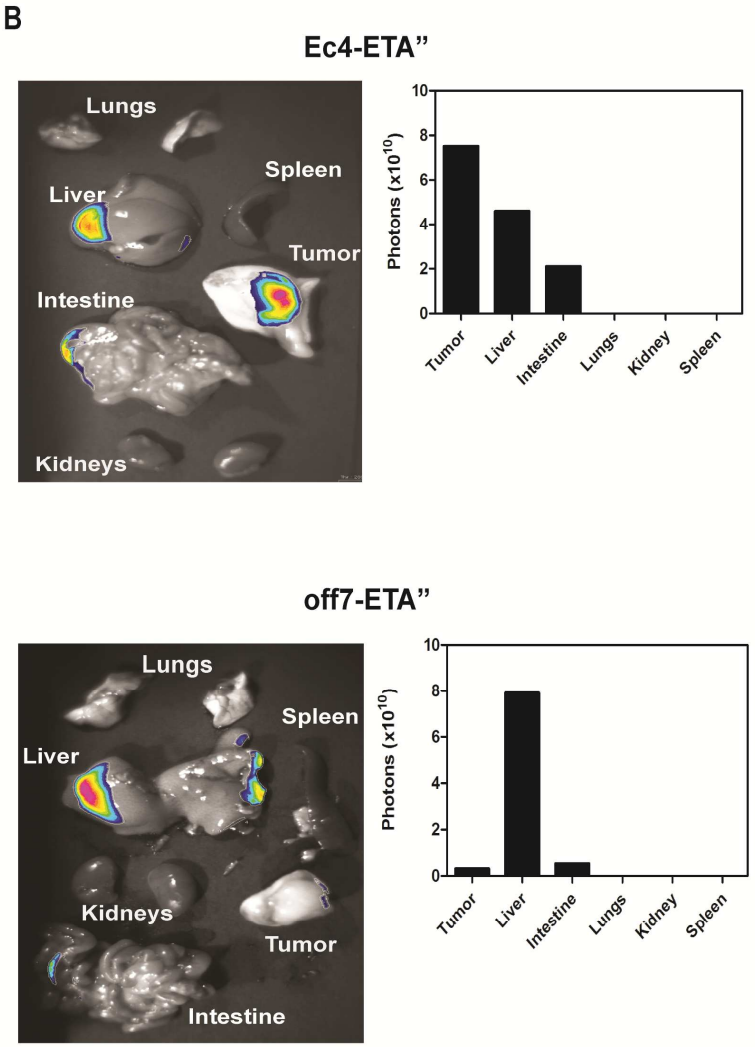



**Figure 4.** Tumor localization and organ distribution of Cy5.5-labeled Ec4-ETA" and the off7-ETA" control fusion toxin detected by *in vivo* fluorescence imaging. Mice bearing s.c. HT29 tumor xenografts were intravenously (i.v) injected with 30 μg of Ec4-ETA” or off7-ETA" conjugated with the fluorescent dye Cy5 (n=3 for each group). **A.** *In vivo* images were acquired 6, 24, 48, 72, and 96 h after injection using the NightOWL II LB891 imaging system with an exposure time of 60 s. For colocalization of the fluorescent image on the animal body, gray scale and pseudocolor images were merged. ND: not determined. **B** *Ex vivo* analysis of fluorescence intensities from isolated tumors and organs 48 h after injection of Cy5.5-labeled Ec4-ETA" or off7-ETA" using a fiber optic device. Samples of one representative mouse of each group are shown.

## Anti-tumor effect of Ec4-ETA"

To investigate how the favorable tumor localization of Ec4-ETA" translates into therapeutic efficacy, its anti-tumor effect was also evaluated in athymic mice bearing established s.c. HT29 tumor xenografts. In one group, mice received 3 doses of 30 µg Ec4-ETA" (Ec4-ETA" 30/3) on days 1, 3 and 5. In a second group, 6 doses of 20 µg Ec4-ETA" (Ec4-ETA" 20/6) were administered on days 1, 3, 5, 9, 13 and 15. Control mice received PBS or 3 doses of 30 µg off7-ETA" (off7-ETA" 30/3) on days 1, 3 and 5.

As shown in Figure 5A, tumors of control mice treated with PBS grew rapidly until the end of the observation period. In contrast, in all mice treated with Ec4-ETA" tumor growth was strongly inhibited. Tumors of mice treated with the Ec4-ETA" 30/3 schedule almost completely disappeared after the last injection, but started to regrow when treatment was discontinued. Nonetheless, in this group 2 of 11 mice (18%) showed complete regression, defined as non-detectable tumor or no tumor regrowth for more than 45 d. The anti-tumor effect of the Ec4-ETA" 20/6 schedule was even more pronounced, resulting in 2 of 5 mice (40%) with complete regressions. Treatment with the control fusion protein off7-ETA" 30/3 had no effect on tumor growth compared to mice treated with PBS.

To better discriminate between the response rates in the various groups, Kaplan-Meier curves were plotted with an end point defined as a tumor size > 100 $mm^3$. As shown in Figure 5C, all control mice treated with PBS or off7-ETA" developed tumors > 100 $mm^3$ already 9 d after the start of treatment. At this time point, all mice in the Ec4-ETA"-treated group had tumors clearly below this size. On day 31, 18% of mice treated with Ec4-ETA" 30/3 and 60% of mice treated with Ec4-ETA" 20/6 still showed tumors < 100 $mm^3$, indicating that treatment with the Ec4-ETA" 20/6 schedule was more effective in tumor control than with Ec4-ETA" 30/3. At the end of the experiment (day 31), the average size of tumors was significantly reduced from 1005 +/- 275 $mm^3$ (PBS group) to 189 +/- 123 $mm^3$ in the Ec4-ETA" 30/3 treated group ($P < 0.05$) and to 95 +/- 79 $mm^3$ in the Ec4-ETA" 20/6 treated group ($P < 0.05$). This reflects a reduction in tumor volume of 81% and 91%, respectively. The favorable effect of the Ec4-ETA" 20/6 treatment is also reflected in the 40% complete regressions (see above). No

difference in tumor size was found in mice treated with off7-ETA" 30/3 compared to PBS.

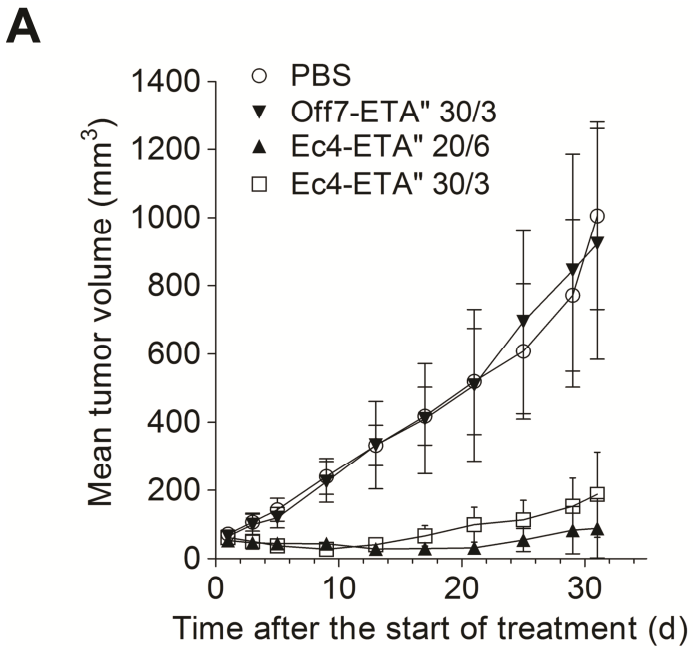



**Figure 5** Anti-tumor effect of Ec4-ETA" in athymic mice. **A.** Mice bearing s.c. growing HT29 tumor xenografts of 50 to 100 $mm^3$ in size received tail-vein injections of either 3 x 30 µg Ec4-ETA", 3 x 30 µg off7-ETA" control, 6 x 20 µg Ec4-ETA" or PBS as vehicle control. Tumor growth was monitored by caliper measurement during the course of 31 days. Data represent the mean tumor volume ± SD of 5 to 11 mice per group.

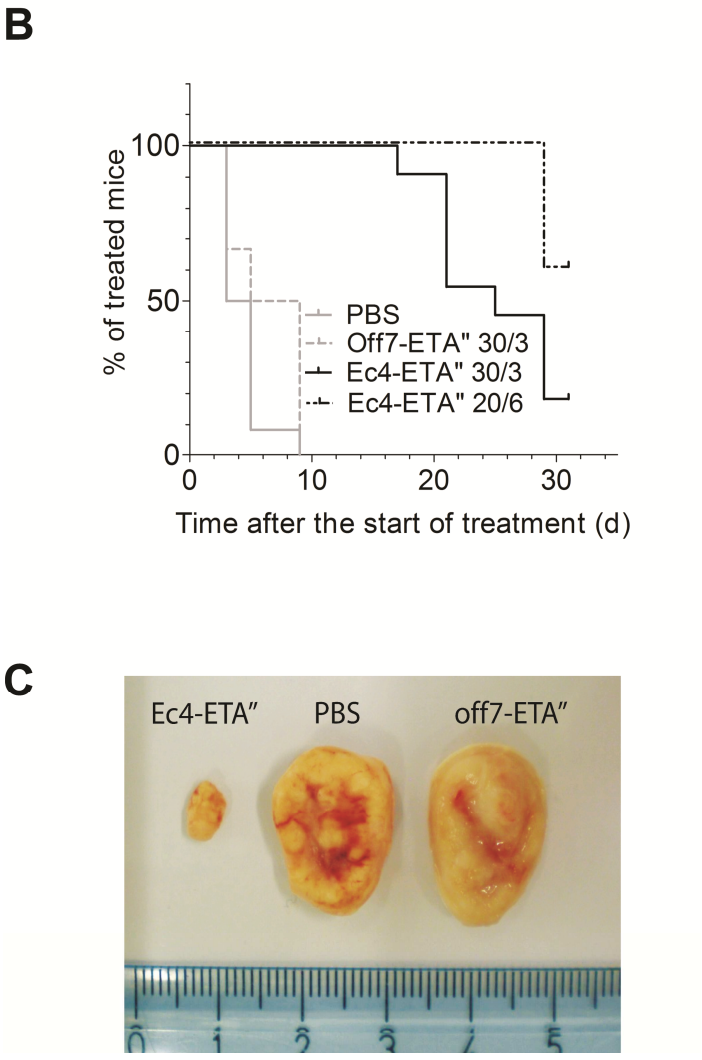



**Figure 5 B.** Kaplan-Meier survival curves with an endpoint defined as tumor volume of 100 $mm^3$. The curves show the percentage of treated mice in each group in which tumors did not exceeded 100 $mm^3$ in size at the various time points after the start of treatment. **C.** Pictures of representative tumors isolated from mice of the different treatment groups

## Toxicity of Ec4-ETA"

To determine treatment-related unspecific toxicity upon Ec4-ETA" and off7-ETA" administration, mice were monitored for weight loss, dehydration and signs of distress (apathy, hyperalgesia and ungroomed appearance) throughout the course of the study. Figure 6 shows that all treatments were well-tolerated and after reversible marginal weight loss after the third injection of Ec4-ETA" 30/3 no further signs of toxicity were observed. Furthermore, to assess liver toxicity as a frequent dose-limiting side effect of ETA fusion toxin therapy in patients, blood from 2 PBS-treated mice and 4 mice receiving 3 doses of 30 µg Ec4-ETA" was collected and analyzed for activity of the liver transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT). As shown in Table 1, liver toxicity could be excluded for this treatment schedule as there was no elevation of ALT and AST activity in the plasma of Ec4-ETA"-treated compared to PBS-treated mice.

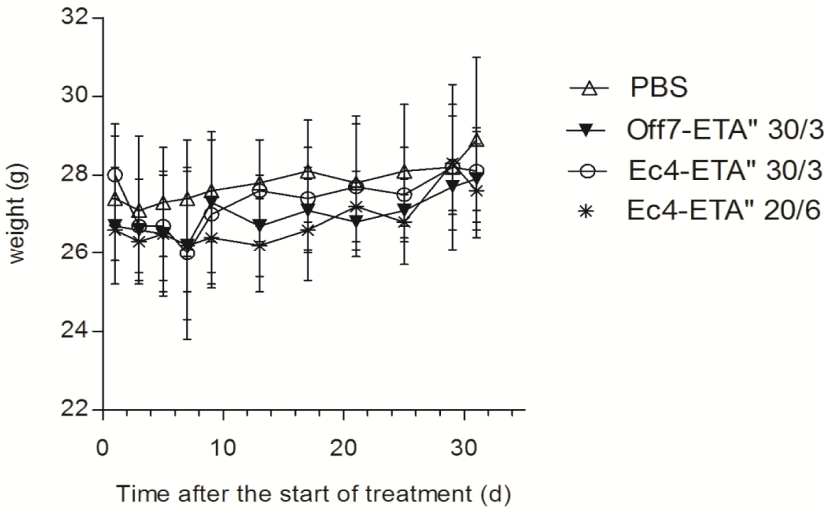



**Figure 6.** Average weight of mice upon treatment with different dose schedules of Ec4-ETA", or off-7-ETA" or with PBS control. Animals were weighed three times per week during the whole experiment to monitor treatment-related toxicity.

**Table 1**. Liver transaminase activity in the plasma from mice treated with Ec4-ETA"

| | **Treatment*** | | | | | |
|---|---|---|---|---|---|---|
| | **Ec4-ETA"** | | | | **PBS** | |
| | Mouse1 | Mouse2 | Mouse3 | Mouse4 | Mouse1 | Mouse2 |
| ALT U/l | 34 | 84 | 57 | 41 | 134 | 40 |
| AST U/l | 141 | 200 | 138 | 85 | 177 | 62 |

*Mice received 3 x 30 μg of Ec4-ETA" or PBS i.v. Activity of the transaminases was measured 24 h after the final injection.

## Discussion

Chemotherapy still has remained the mainstay of cancer therapy. The great majority of approved treatments have no inherent specificity for tumor cells but attack all dividing cells. More recently many investigations have been carried out to couple a toxic principle to a recognition function. Immunotoxins based on *Pseudomonas aeruginosa* exotoxin A (ETA, also termed PE), particularly its truncated variant carrying a C-terminal KDEL peptide (ETA"), have been generated against various cell surface receptors and extensively tested in preclinical and early clinical studies (4, 31). Most of them use a single-chain fragment (scFv) or a disulfide-stabilized dsFv fragment of antibodies as targeting moiety. We previously also reported on the potent anti-tumor effect of an EpCAM-specific scFv-ETA" immunotoxin (10), which is currently under phase II clinical investigation (8).

Commonly used antibody Fv-based formats, however, are difficult to produce in high amounts when compared to other proteins and are often notoriously aggregation-prone (23-24, 32). ScFv and dsFv fragments used for tumor-targeted fusion proteins are commonly expressed in the periplasm or need to be refolded after expression in inclusion bodies.

Here, we investigated another class of highly advanced binding molecules, Designed Ankyrin Repeat Proteins (DARPins), for targeted delivery of ETA" to tumor cells *in vitro* and *in vivo*. DARPins can be selected for high affinity which is a major requirement for efficient tumor targeting (33), their inherent robustness allows easy conjugation with various types of effector molecules, and pharmacology and tumor targeting properties can be easily modulated, e.g. by site-specific PEGylation. Therefore, we are not limited to the simple fusions described here, the molecules are robust enough to allow efficient production of more complicated constructs.

We are expressing the DARPin-ETA" fusions in the cytoplasm of *E. coli* and find that these fusion proteins are produced in soluble form. Even though the ETA part contains two disulfide bonds, which appear to be beneficial in the internalization process (34), we find that they have been formed almost quantitatively after the protein has been purified, possibly by air oxidation. Thus,

we can use the convenient production of DARPins in the *E. coli* cytoplasm also with the DARPin-ETA" fusion proteins.

A major challenge for the application of immunotoxins is the choice of targets which provide sufficient tumor specificity and, at the same time, promote intracellular delivery of the payload. Most tumor-targeting with fusion toxins has been studied in hematopoietic malignancies, but even there, only one (denileukin diftitox, Ontak®) finally received FDA approval (35).

EpCAM is overexpressed in many solid tumors and on basolateral cell surfaces of some normal epithelia (14, 17) where it is, however, poorly accessible to circulating anti-EpCAM antibodies (16). Recently, EpCAM was also identified as a marker of cancer-initiating cells in colon (20), breast (21) and pancreatic cancers (22). Since cancer stem cells respond poorly to standard therapy and thus are largely responsible for treatment failure, their elimination must be a prime objective for all kinds of innovative cancer therapy. Thus, from a therapeutic point of view, EpCAM is more interesting as a docking site for targeting ligands delivering external effector molecules. In fact, EpCAM efficiently mediates internalization of bound ligands by receptor-mediated endocytosis and thus perfectly matches the need of anti-cancer agents acting on intracellular targets, such as protein toxins, chemotherapeutic agents, and antisense compounds (10-12).

Recently, we have described for the first time the production and biochemical characterization of EpCAM-specific DARPins, and a first generation binder was used to successfully deliver therapeutic siRNA into tumor cells in the form of mono- and multivalent binders fused to highly charged protamine (13). Subsequent affinity maturation efforts then resulted in DARPin Ec4, which displayed affinity to EpCAM in the subnanomolar range and excellent biophysical properties (Martin-Killias et al., in preparation).

We measured good tumor localization of Ec4-ETA", but not of the control fusion off7-ETA" by *in vivo* fluorescence imaging upon systemic administration in a HT29 colon carcinoma xenograft model. These data showed that localization in the tumor was specific and dependent on EpCAM binding with peak tumor accumulation 48-72 h after injection.

Fluorescent imaging records the distribution of molecules at any particular time point, while the previously used residualizing label $^{99m}Tc(CO)_3$ (33) gives an integral localization since the begin of injection, since any label will accumulate at the site of cellular internalization. This probably accounts for the difference in kidney accumulation, which is seen to decay rapidly when measured by fluorescent imaging but not by $^{99m}Tc(CO)_3$ radioactivity. While immunotoxins (scFv fragments or DARPins fused to a protein toxin such as ETA) would be expected to not fall in an ideal MW range for maximum accumulation (33), we see very encouraging enrichments and therapeutic effects already with the constructs described here, and there may be even better effects with different molecular formats.

In the present study, Ec4 was fused with ETA" to assess for the first time the potential of a rationally engineered DARPin for tumor targeting and therapy. The fusion protein could indeed be well produced in soluble form in *E. coli* (at about 80-fold higher yield from shake flasks than our previously described scFv-ETA" fusion toxin (4D5MOCB-ETA) (10), could be easily purified and was stable and resistant to aggregation. This will facilitate subsequent scaling up of the production process required for clinical trials.

We found that the high affinity binding of Ec4 to intact cells was fully preserved upon fusion with ETA" and that Ec4-ETA" showed extremely high and specific *in vitro* cytotoxicity against EpCAM-positive tumor cells of various histotypes with $IC_{50}$ values of 0.005 pM. This is remarkably low compared to other immunotoxins (36-38) and likely mirrors its high stability, affinity and efficient internalization by receptor-mediated endocytosis. These data, combined with efficient tumor targeting, indeed translated into potent anti-tumor effects at well-tolerated doses with some mice showing complete regression of their tumors. The repeatable response measured after repeated injections further suggests that tumors retained a stable EpCAM expression profile and that antigen loss did not occur during treatment.

A phase II study with a previously reported EpCAM-specific immunotoxin consisting of ETA" fused to a scFv antibody is ongoing and will be completed soon (8). Based on the findings of this study, it is tempting to speculate that DARPins like Ec4 will replace the antibody fragment as cell binding ligands also in

forthcoming generations of tumor-targeted fusion toxins as well as other drug delivery systems.

In summary, we describe for the first time the generation and preclinical evaluation of an EpCAM-specific fusion toxin consisting of a high-affinity DARPin (Ec4) and ETA" as a catalytic biotoxin. We provide evidence for its potent activity against various tumor cell types *in vitro,* and its favorable tumor localization and anti-tumor activity *in vivo*. The advantages of DARPins enabling high yield expression, resistance against aggregation, and stability also in the form of fusion toxins in conjunction with a highly selective tumor-associated target like EpCAM, opens new avenues for the generation of rationally designed protein therapeutics with outstanding efficacy.

## Acknowledgements

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**Chapter 3**

# General conclusions and future perspectives

# General conclusions and future perspectives

The improvements in translating discoveries in molecular biology into diagnostics for and treatments of cancer have significantly broadened the spectrum of available therapeutics. Cancer treatments are shifting from chemotherapeutic drugs or radiotherapy towards treatments directed to the individual tumor type. Treatments can now be based on cancer-specific biologic mechanisms and, more importantly, can be tailored to the molecular specificity of a tumor type. The advantage of tumor targeted approaches is the reduction of systemic toxicity through selective delivery of drugs to the tumor. In addition, these approaches can provide powerful anti-cancer agents acting by alternative mechanisms, thus being effective on tumors that do not respond to or have acquired resistance to traditional therapies (1). One such concept is represented by immunotoxins. They mostly make use of monoclonal antibodies or fragments thereof for the specific delivery of a toxin to tumor cells (2). In this context, only the binding site of the antibody is required to localize the cytotoxic moiety in tumors. As tight binding is not restricted only to antibodies, alternative binding scaffolds with more favorable properties compared to antibodies, such us robustness, ease of modification and cost-efficient production could be beneficially used as targeting moiety.

The concept of alternative binding proteins, based on new scaffolds with superior properties, has undergone rapid development and has been proven by different approaches (3). DARPins are a promising alternative to antibodies and have demonstrated to be valuable tools for a wide range of applications. *In vitro* selection techniques such as ribosome display and phage display have allowed the selection of high-affinity DARPins against a variety of different targets and their functionalities are explored in numerous assays. The first binders were obtained using ribosome display and specifically recognized maltose binding protein and two eukaryotic kinases (4). Since then, there has been an increasing number of selected DARPins towards a broad range of target proteins. E.g., several inhibitors have been selected from DARPin libraries demonstrating a potential for modulation of protein function *in vitro* and *in cellulo* (5-6). High affinity binders against members of the human mitogen activated protein kinase (MAPK) family were selected previously (7) and their potential applications as

intracellular inhibitors are currently being studied (P. Parizek, personal communication). Other examples of enzyme inhibition are DARPins capable of inhibiting the tobacco etch virus protease, a plant pathogen. A highly specific caspase-2 inhibitor was selected showing an allosteric mechanism of inhibition (8). DARPins were also successfully selected against CD4; here, a specific DARPin could potentially block HIV entry (9). Targeting the immunoglobuling E receptor, DARPins could prevent the release of proinflamatory mediators (10). In the field of oncology, several DARPins have been selected against a variety of relevant targets. The first example described DARPins binding to the tumor antigen HER2. These were successfully used for staining of sections of breast carcinoma and it was possible to evolve them to picomolar binders (11-12). In addition, bivalent HER2 binders (Tamaskovic and Jost et al., in preparation) and EGFR-specific DARPins (Boersma et al., in preparation) are showing promising cytotoxic effects. Taken together, these examples illustrate the wide applicability of DARPins.

In the first part of my thesis (chapter II), I focused on the selection of DARPins against the extracellular domain of the clinically validated tumor-associated antigen EpCAM. EpCAM is a particular interesting target due to its high expression in a plethora of tumors. The accessibility of its extracellular domain as well as its rapid internalization makes it an ideal target for the delivery of anti-tumor agents (13). To select DARPins specifically recognizing EpCAM, both phage and ribosome display techniques were used. The application of both selection procedures with different combinatorial DARPin libraries was particularly effective to select a variety of EpCAM binders. The best binder in terms of affinity, Eph1, has a particular deletion of two amino acids. This deletion appears to be important for tight binding to EpCAM since correcting it markedly reduced EpCAM binding. This binder shows a self-association tendency probably due to particular features of its sequence as analyzed by ROSETTA (14). Using affinity maturation, six improved second generation EpCAM-specific DARPins were generated, four of which were derived from EPh1. The evolved EPh1-derived binders have the same randomized positions as well the characteristic two amino acid deletion, but four to six framework mutations. They were monomers, indicating that the dimerization or oligomerization tendency was abolished by the framework mutations introduced during directed evolution. The high affinity of these EpCAM-specific DARPins, a major requirement for efficient tumor targeting, in conjunction with the favorable properties of EpCAM as a

molecular target, provided an ideal starting point for the generation of fusion proteins delivering a toxic payload to EpCAM-expressing tumors.

In the second part of my thesis (chapter III), the focus was on the generation of cytotoxic fusion proteins using one of the characterized EpCAM-specific DARPins (Ec4). Ec4 was genetically fused to a truncated form of the potent bacterial toxin *Pseudomonas* exotoxin A (ETA"), and analyzed for its cytotoxic effect on tumor cells. Ec4-ETA" was expressed in soluble form in *E. coli* and purified with at least 80-fold higher yields as its counterpart, an EpCAM-specific scFv_ETA fusion protein expressed in the periplasm (15). This remarkable increase in yield can be attributed to the favorable properties of DARPins in means of expression and purification. Consequently, using a DARPin as targeting moiety fused to ETA" was highly advantageous.

To demonstrate that cytotoxicity was mediated by receptor-specific uptake, Ec4-ETA" was tested in a variety of tumor cell lines of different histological origin. Ec4-ETA" was highly cytotoxic to cells expressing EpCAM but not to EpCAM-negative cells. The cytotoxic effect was dependent on EpCAM binding as it was markedly reduced when the cells were preincubated with Ec4 as a competitor but not with an unspecific DARPin. In addittion, unspecific DARPin-ETA" fusions showed cytotoxic effects at much higher concentrations.

To translate the success of a targeted agent from *in vitro* or *in cellulo* to *in vivo* depends in part on the ability of the targeted drug to reach and penetrate the tumor. The distribution of a drug in a tumor depends on several factors and is a complex process involving extravasation from tumor capillaries, diffusion through the tumor interstitium, systemic clearance and drug degradation in the tumor tissue (16). Recently, it was demonstrated that for small proteins like DARPins tumor accumulation was proportional to affinity (17). Small proteins accumulated rapidly into tumors but required high affinity to be retained there as unbound molecules were cleared from the tumor rapidly. In contrast, larger molecules, in a size range larger than the kidney filtration cut off, can achieve good tumor accumulation with less affinity. Their decreased vascular extravasation can be compensated by a decrease in systemic clearance, producing a net increase in tumor uptake (18) . Intermediate molecules, such as DARPin-toxin fusions, are in an unfavorable situation. Their capillary extravasation to tumors is diminished but they are probably still partially

eliminated by the kidneys (17-18). Nevertheless, Ec4-ETA" showed specific tumor accumulation in a mouse xenograft model for human colon adenocarcinoma with a peak 48-72 h post injection. Tumor accumulation was translated in antitumor activity demonstrating the ability of Ec4-ETA" to remain stable in serum, gain access into tumors and cells, and inhibit tumor growth. Both dose treatments, 30 μg given 3 times (30/3) or 20 μg given 6 times (20/6) of Ec4ETA", were effective to significantly delay tumor growth and some mice showed complete tumor regressions. No systemic toxicity was observed as evaluated by mice weight data and liver enzymes determination. The percent of treatment/control (PBS) average tumor volume calculated at the end of the experiment (day 31) was 92 % for the unspecific DARPin_toxin, off7-ETA"; 19% for Ec4-ETA" 30/3; and 9% for Ec4-ETA" 20/6. This indicated that more regular doses were more effective to reduce tumor growth. Further studies will be needed to extend these findings to other cell lines and models.

Although both dose schedules delayed tumor growth significantly during treatment, when complete regression was not achieved, tumors resumed growth around one week after the treatment finished. Probably Ec4-ETA" effectiveness was compromised because it was not able to access all tumor cells. Those cells that were not targeted by the DARPin-toxin were able to start growing again when treatment was discontinued. Other dose schedules and molecular formats should be evaluated to achieve more effective tumor penetration.

It must be noted that two major problems should be addressed when working with ETA based immunotoxins independent of the targeting moiety: ETA immunogenicity and unspecific toxicity, to which liver and endothelial cells are the most susceptible. However, ETA-based immunotoxins are under further development to reduce these unspecific side effects and these approaches should also be applicable to DARPin-ETA fusions. Engineering of less immunogenic ETA variants, PEGylation, the use of immunosuppresive agents, and inhibition of TNF-alpha production in the liver are some of the approaches proposed for the improvement of the efficacy of immunotoxins (19-23).

The high number of preclinical and clinical trials carried out with a variety of immunotoxins (24) demonstrates that immunotoxins are suitable as potent anti-cancer agents.

# Future perspectives

DARPins advantages, specifically their robustness combined with cost-efficient production, make them ideal tools to be used for both diagnostic and therapeutic applications. For the first time, EpCAM-specific and high affinity DARPins were selected and characterized. As a proof of principle, this project has demonstrated the suitability of DARPins for the efficient delivery of ETA toxin to tumors *in vivo*. However, the application of the selected EpCAM-DARPins is certainly not restricted to the delivery of this particular toxin. There are different classes of payloads that have been used in targeted therapies to mediate cell killing, including toxins (as described here), cytokines, radionuclides, chemotherapeutics drugs and enzymes. Each of these approaches present advantages and disadvantages. Nonetheless, the specificity of these fusion proteins depends on the targeting moiety and DARPins are well suited to be equipped with such effector molecules. Numerous other applications can be envisioned such as *in vivo* tumor imagining (25) or tissue staining (11). Different molecular formats could be easily engineered such as dimers or multimers with different specificities which might be useful to bring reaction partners together (26). In addition, EpCAM-DARPins might be also helpful for the determination of EpCAM structure in crystallization trials (27). Further applications are currently being investigated, such as the generation of bivalent EpCAM-DARPins, a second generation of Ec4-ETA" with improved tumor specificity, and the development of nanoparticles in which Ec4-ETA" is encapsulated.

It is clear that in a time of rising healthcare costs, the cost of a therapeutic agent is a major point of concern. In this respect and due to their improved biophysical properties the use of DARPins as therapeutic agents will be of great advantage facilitating the upscaling of the production process and providing an economical advantage. The results obtained here strongly encourage further exploration of EpCAM-DARPins in therapeutic applications.

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